

# Ethics and the human genome

Efforts to reach agreement on the use of genetic information will be frustrated while the world — and people's capacity to live with truth — is changing as quickly as at present.

LAST week's meeting at the National Institutes of Health on the ethics of the promised better understanding of the human genome may have failed to win consensus on an international treaty regulating the use made of genome data (*Nature* 351 507; 13 June 1991), but that is neither surprising nor shameful; it is too early. Yet the underlying issues are important. So much has been recognized from the outset by the chairman of the US project, Dr James Watson, who used to talk of setting aside some proportion of the project's funds for the study of these questions. But the need now is not of money, but of talk, which is relatively cheap but none the less important on that account.

Not that there has been none. Over several decades of genetic counselling, physicians have been acutely conscious of the sensitivity of personally significant genetic inferences about their patients. It has become the practice to tell presumed carriers of, say, a thalassaemia defect of the chances that children will be affected, leaving it to the patient and his or her spouse to decide whether to have children. Experience is that potential parents are more ready than naive expectation would suggest to be told the truth. But that is only part of the battle. Even the prudence of genetic screening for cystic fibrosis on the basis of the most common of the causative genes now recognised remains in dispute, given counsellors' fear that reassuring advice may be falsified by events. And what is to be said to the presumed homozygotes for Huntington's disease? Will knowledge of their likely fate be helpful while there is no prospect of intervention and palliation?

The diffidence of physicians and counsellors in such daunting circumstances is understandable, but may be misplaced. Genetic counsellors have been more often surprised by people's willingness to know than assailed as the carriers of unwelcome news because the world is changing. In a way, it is a triumph for the general understanding of science: people accept that the DNA they carry may predispose them or their offspring to what used to be called bad luck. (The admirable fortitude of a large proportion of those infected with HIV illustrates, in a different connection, the same tendency.) The absorbing questions are how far the wish to know will go, and what the consequences will be.

At least two different, and apparently contradictory, influences are at work. First, there is the business of the use of personal genetic information to calculate people's risk of calamity later in life. Second, there is the eugenic inclination stemming from people's wishes that their descendants should be genetically as well-endowed as possible. Both

tendencies long predate the Human Genome Project. Insurance companies were enquiring about family histories of heart disease long before the recognition of genes linked with cardiac trouble, while traditional and mostly unsound marriage-broking made no secret of its eugenic ambitions. The genome projects will simply provide a more systematic way of setting about tasks such as these (as well as, in the end, providing better understanding of external influences on largely genetic processes).

Geneticists, like genetic counsellors, are for the time being over-diffident on both counts. They hold that whatever comes from the genome projects should not be "exploited" by the commercial insurance companies or by people seeking eugenic improvement. But neither position is sustainable. A rule that insurance companies should not seek genetic information about potential policy-holders would probably be unenforceable, would be unjust to those free from defect and would probably be unconstitutional in most advanced countries. Social policy should rather aim at requiring companies not to cancel policies when relevant genetic information comes to light (as well as indexing them against inflation).

The eugenic issue, to which the present practice of amniocentesis followed (sometimes) by abortion is a crude approach, is similarly unstoppable in the long run. One of the potential benefits of the genome projects is the understanding whether particular genes acting in concert are likely to have valuable consequences. Can prenatal diagnosis of such combinations be effectively prevented? And can even the manipulation of the human genome be prevented in the long run, whatever national legislation may say about today and tomorrow? To raise these questions is not to rubbish the genome projects, but is simply to say that they deserve discussion. □

## Dioxin damages denied

Monsanto has won a famous legal battle against a frivolous and irresponsible lawsuit.

A TANK car containing 19,000 gallons of wood preservative and a teaspoon of dioxin sprang a leak near the small town of Sturgeon, Missouri. Sixty-five citizens proceeded to sue the railroad and Monsanto, the manufacturer of the preservative contaminated with dioxin. Exposure to dioxin, they claimed, was causing them headaches, insomnia and depression. The railroad settled out of court, but Monsanto said "Nothing



doing", deciding instead to go to trial. Given the penchant of US juries to go after big corporations (especially those accused of causing environmental damage), Monsanto took a big risk that paid off only last week, when an appeals court threw out a jury verdict that the company should pay more than \$16 million in punitive damages.

The original trial, known as *Kemner* (for one of the plaintiffs) *v.* Monsanto, lasted for 44 months, setting a record as the longest jury trial in US history. More than 200 witnesses testified. More than 6000 exhibits were offered in evidence. When it was all finally over, the jurors concluded that there were no scientific data to support the plaintiffs' claims that they had been injured by dioxin. But the same jurors did not let science or commonsense cloud their judgement when they slapped the \$16 million penalty on Monsanto.

Last week, the Missouri appellate court, evidently better endowed with commonsense, put things right when it ruled that the jury could not have it both ways. If there is no evidence of injury, there can be no justification for punitive damages. The wonder is that the case got that far in the first place. That the US tort system has run amok, as this case illustrates, is nothing new. But the depth of scientific ignorance and hostility towards scientifically based industry revealed by the jurors' action is frightening, leaving the suspicion that they might actually have believed the claims of dioxin injury even in the absence of data. Monsanto could probably have saved itself millions of dollars by settling case out of court, but would then have perpetuated the idea that any level of exposure to a toxic chemical, however small, is hazardous to people's health. □

## Policy by stealth

Science has become a British election issue, but as yet in an unreconstructed fashion.

If proof were needed that Britain is in the throes of an undated general election campaign, the manner in which the Conservative or government party announced its policy for science and research last week provides it. Weeks ago, the Labour Party had booked a lecture hall at the Royal Institution for 4 June, and had announced that Mr Neil Kinnock, its leader (called "Leader") would introduce Labour's much leaked science policy then. So, the Conservatives seem to have calculated, would it not be a splendid wheeze to announce their own policy for science the previous evening? That, in the event, is what happened, but with such haste that many (this journal included) did not learn what was afoot in time, and thus reported only the contents of Labour's document (see *Nature* 351, 512; 13 June 1991). What follows may make amends (but only partially).

What the Conservative Party says of its record over twelve years is not in itself objectionable: much of it is also factually true. At the school level, the government has indeed made science a part of the national curriculum. In basic research, there has indeed been an increase of 22 per cent (allowing for inflation) in funds spent through the five research councils,

showing that Mrs Margaret Thatcher's promise to "protect" the science budget has literally been kept. And it is also true that "our scientists continue to excel" (witness Nobel Prizes, bibliometric indices and such innovations as DNA fingerprinting, typing of the virus responsible for seal kills in 1987 and 1988 and the discovery of the Antarctic ozone hole).

True, there is a fly in the ointment — government spending on civil science has fallen as a proportion of gross domestic product over the past decade, but (the Conservatives say) that reflects both an increase of GDP greater than 22 per cent and the government's decision (half-way through the decade) not to support "near-market research". So why is the government's record so widely (although not generally) pilloried?

The polemical part of the Conservatives' anticipatory response to Labour rests on the charge that the Labour Party stands for massive public spending and the central direction of affairs. (The wider political argument in the general election in the next twelve months may be decided by the British electorate's view of whether Labour has, as it claims, given up these old hankerings.) Curiously, the Conservative Party overlooks its own proclivities for telling others, especially academics and researchers, how they should conduct themselves.

In the history of British research policy, the 1980s stand out as the decade in which research councils were given specific marching orders (on matters such as support for information technology), budgets were changed to accommodate government prejudices (for the Antarctic, against social research), when research councils were coerced into thinking up schemes for collaborative research with industry more quickly than they could think them through and when the whim for directed administrative change (in universities as well as research establishments) led to the waste of funds, the needless (and expensive) retirement of useful people and the distraction and demoralization of many able people. The 1980s in Britain are a vivid proof that an intellectual enterprise can be ruined without starving it of funds, but simply by kicking it in the teeth often enough.

Has this lesson now been learned? The Conservatives do not directly acknowledge that the government's predecessor was outrageously a busybody in dealing with the British research community, but there is a hopeful sign in the subtitle of the Conservative science policy — the phrase "the cultural revolution". The text, among others things, makes the valid point that innovation flourishes only when the wider economic climate is welcoming, at least implying that if it were reelected, it would not go about telling researchers that it is their "bounden duty" (one of Mrs Margaret Thatcher's little phrases) to make the country prosperous.

Instead, the Conservatives say they want to bring about in British industry "a revolution in cultural attitudes towards innovation". High time. The irony is that their government is struggling to right an economy in which last decade's entrepreneurs are being forced to the wall in great numbers. The memory of this will not be quickly exorcised even if prosperity is restored before the general election. □



# Japan's Human Genome project takes shape

- New DNA analysis centre planned
- Questions remain about coordination

## Tokyo

THE Science and Technology Agency (STA) is planning to set up a new DNA analysis centre supported by private industry as part of Japan's effort to map and sequence the human genome. STA's move comes as the agency and two other ministries manoeuvre to cut up the pie of government-sponsored human genome research in Japan.

The centre, if established, will be Japan's third, following similar initiatives by the Ministry of Education, Science and Culture and the local government of Chiba prefecture (*Nature* 349, 640; 1991).

Some experts question, however, whether all the various government-backed efforts will be combined into an effective whole.

STA's proposed DNA analysis centre is a top priority for submission in August in the agency's budget request for next year, according to people familiar with the agency's plans. Masato Chijiya, director of STA's life science division, admits that such a plan is in the works, but he is hesitant to discuss details at this stage because the agency's budget is still under internal discussion and it has not yet been presented to other ministries.

The education ministry, STA and the Ministry of Health and Welfare, which has a project to sequence disease-related human genes, have only recently come close to agreement on how to divide this year's budget for human genome research. Chijiya says the figures have yet to be firmly fixed, but the total outlay is expected to be about ¥2,000 million (\$14 million). STA will spend ¥1,203 million (\$8.6 million), and the two ministries will get about ¥400 million (\$2.9 million) each.

These figures are deceptively low, says Kenichi Matsubara, a key organizer of Japan's human genome research. The education ministry's budget covers only grants and money for equipment, Matsubara points out, unlike budgets for human genome research in the West, in which salaries are usually included. And whereas that ministry has about 60 researchers involved in human genome research, STA is only supporting about 20. The budgets also exclude outlays for construction of new buildings, which are hidden elsewhere in the national government budget.

The education ministry is in the process of establishing its own new human genome analysis centre at the Institute of Medical Science of Tokyo University (*Nature* 349, 360; 1991). The centre, to be headed by Minoru Kanehisa, a computer and database

expert from Kyoto University, will get under way this fiscal year with a complement of four researchers in a 'genome database division'. It is hoped that Tokyo University centre will eventually have about 24 researchers, Matsubara says.

In a move independent from all the national government projects, the local government of Chiba prefecture has also announced that it will establish a new DNA analysis centre in 1993 in a science park for private companies (*Nature* 349, 640; 1991). The centre is expected to concentrate on sequencing DNA and will probably be manned largely by technicians.

Chijiya says that the planned STA centre, if realized, will also probably have a "large number of technicians". And he confirmed reports by scientists connected to the agency that STA is hoping to win private-sector backing for the centre. Several such semi-private institutes have been established in Japan recently by other ministries and they are among the nation's best-funded and best-equipped research organizations.

The planned STA centre will adopt a 'top-down' approach to look at the human genome as a whole, Chijiya says, in contrast to researchers supported by the two ministries, which will use a 'bottom-up' approach

and focus on specific genes. The STA centre will concentrate on mapping the human genome until advanced sequencing technology is developed.

Under a separate budget, STA is hoping to start a project in fiscal 1992 to develop a next-generation automatic sequencing system at the Institute of Physical and Chemical Research where an automatic sequencer, HUGA, has just been unveiled (see below). In the meantime, HUGA will probably be used at the planned centre to sequence DNA clones that have already been mapped, Chijiya says.

But Yusuke Nakamura in the privately-funded Cancer Institute in Ikebukuro, Tokyo, who is among those who will soon receive grants from STA for human genome mapping, is sceptical about whether all these government-backed efforts will fit into a well-coordinated whole. Japanese scientists should get together to organize the project themselves rather than relying on government bureaucrats, he says, but Japan's human genome researchers have no one place or organization under which to meet.

Matsubara is vice-president of the Human Genome Organization (HUGO), a large group of scientists from around the world which was established to coordinate international research on the human genome project. Matsubara says he is encountering considerable difficulty in getting financial support for HUGO in Japan because of government red tape that inhibits the formation of privately funded foundations, and he predicts that it will probably be quite a while before HUGO gets financial support in Japan.

David Swinbanks

## The new automated DNA sequencer

RESEARCHERS at the Institute of Physical and Chemical Research have unveiled Japan's first fully automated system for the analysis of DNA. Nicknamed the Human Genome Analyzer (HUGA), the robot-run system is expected to play an important role in Japan's efforts to sequence the human genome.

HUGA, the end product of a project initiated by Akiyoshi Wada of Tokyo University in 1981 with the backing of the Science and Technology Agency and several private companies, consists of a production line of machines that carry out the different steps of DNA analysis from initial purification to input of the DNA sequence into a computer. Robots transfer microtitre plates and electrophoresis gel cassettes between various parts of the analyser under the control of a supervisory computer system developed at the Institute. This is the first automatic system to cover so many steps of DNA analysis, says Kenichi Matsubara, one of the leaders of

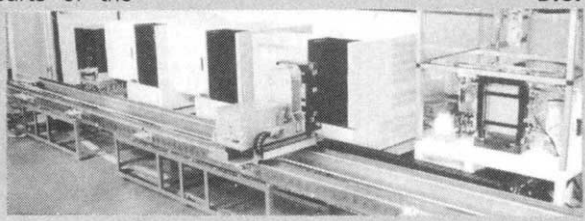
Japanese human genome research.

The analyser can run unattended at night and has a potential raw output of up to 108,000 base pairs of DNA per day. Because of the need for duplicate sequencing to cover damaged parts of a given sequence and to provide controls, the actual output of correct and complete sequences is about 20,000–30,000 base pairs per day.

The Science and Technology Agency is considering setting up a semi-private organization backed by industry that will use HUGA.

But while industry may sell individual parts of the human genome analyser, there are no plans to commercialize the system as a whole.

D.S.





# Back to the future

## Washington

NATIONAL Aeronautics and Space Administration (NASA) officials should brush the dust off their old research and development files, if they are to meet President George Bush's goal of putting Americans on Mars early next century. A high-level panel has concluded that launchers capable of lifting 150–250 tonnes into Earth orbit and a nuclear thermal rocket propulsion system will be needed to mount a manned Mars mission.

Both concepts were explored by NASA in the 1960s, but the projects were abandoned in the early 1970s, after the United States decided not to follow the Apollo Moon programme with further manned space exploration.

Last year, NASA and Vice President Dan Quayle asked an independent Synthesis Group headed by former astronaut Thomas Stafford to scour the space community for technologies useful for a Mars mission (see *Nature* 345, 651; 1990). The group's assignment came after the widespread derision that greeted NASA's own \$500,000 million Mars

plan, published several months after Bush's July 1989 'Man on Mars' speech. At the time, critics said NASA's recommendations were little more than a rehash of the Apollo programme.

The Synthesis Group's report says that nuclear thermal propulsion — in which a nuclear reactor heats a low-weight rocket propellant to high temperature before the propellant is expelled from a rocket nozzle — is "the only prudent propulsion system" for a flight to Mars. Using nuclear thermal rockets could cut the time taken to travel from Earth to Mars and back again from 400 to 320 days.

Stafford's group has produced four outline plans, or 'architectures', for an eventual manned flight to Mars. Each involves initial landings on the Moon, beginning between 2003 and 2005, before an assault on Mars in 2014 or 2016. The plans differ in the amount of science done, in the amount of infrastructure established on the Moon, and in the degree of effort made to exploit the resources of space — for example by generating solar power on the Moon, and relaying this to

Earth using microwave transmitters or lasers. The report does not provide cost estimates for any of the plans.

Even the architecture that places the strongest emphasis on science, calling for complementary manned and unmanned missions to Mars and a near-Earth asteroid, and extensive scientific studies on the Moon, is unlikely to win much support from the US planetary science community. Bruce Murray from Caltech in Pasadena, California, an advocate of manned space exploration, predicts that the Mars mission will be opposed by the vast majority of his colleagues, who feel that spending on NASA's manned programmes is diverting funds from science. The House of Representatives voted earlier this month to fund development of the manned space station Freedom in the 1992 fiscal year, but refused to increase NASA's overall budget from its 1991 level.

Winning congressional approval for the Mars initiative will also be difficult, given the continuing concern over the US budget deficit, although Stafford argued last week that significant funds would not be needed until after spending on many of NASA's other as planned large projects begins to wind down.

Peter Aldhous

## PARTICLE PHYSICS

# Friction continues over costs

## Washington

RELATIONS between US particle physicists and their European counterparts at CERN, the European particle physics centre in Geneva, continue to be strained.

CERN director-general Carlo Rubbia is objecting to overhead charges made to European particle physics working in the United States. The total sum involved is less than \$2 million a year, but the dispute has brought back into the open the nagging rivalry between the Superconducting Super Collider (SSC) laboratory and CERN. The Geneva laboratory has plans to build its own new accelerator — the Large Hadron Collider — to do much the same physics as the SSC.

European physicists working at US particle physics laboratories at Brookhaven National Laboratory, Fermilab and the Stanford Linear Accelerator are charged a fixed percentage, typically about 30 per cent, on top of the cost of equipment ordered from laboratory stores, to cover overhead expenses such as administrative costs. This is standard US practice and not a measure reserved for overseas scientists, but historically CERN has not made similar charges to visiting US physicists.

With the number of US visitors to CERN growing rapidly (see figure) and the US Department of Energy seemingly reluctant to negotiate, CERN's management had become increasingly irritated with the situation. Last week, the Department finally agreed to set up a joint committee with

CERN to discuss the issue, after Rubbia threatened to impose overheads on US scientists. This concession should have calmed matters, but now a briefing document put out by CERN to explain the overhead issue has reopened old wounds at the SSC laboratory.

The document says that "the shadow of the SSC" lies behind the overheads problem. It goes on to complain that Europeans working on the SSC project would be regarded as "mere co-workers", with no capability to make decisions about the project's direction. The document cites the recent decision by Switzerland and Germany not to join the SSC project as evidence of the difficulties — a reference to the withdrawal of these two countries from the consortium to build the now cancelled L\* detector for the SSC (see *Nature* 351, 258; 23 May 1991).

SSC director Roy Schwitters describes CERN's commentary on the background to the overheads problem as "a very disturbing statement". The SSC does want European input on decisions, he says, and senior CERN scientists are represented on the SSC's advisory panels. But in the past, Schwitters says, Rubbia has been reluctant to let CERN physicists come to SSC meetings.

In April, Rubbia and Schwitters appeared to have resolved their difficulties with the signing of an agreement to exchange scientists on a "no gain, no cost" basis. But the latest friction shows that successful co-operation between the two laboratories is still fraught with difficulty.

Peter Aldhous

## AGEING RESEARCH

# Spend to save on health care

## Washington

WITH the population of US senior citizens growing steadily, the United States should increase its spending on ageing research by 50 per cent over the next five years, according to a report from an Institute of Medicine committee. Published last week, the institute's 'National Research Agenda on Aging' asks for an extra \$312 million a year for ageing research, including \$92 million for basic biomedical research that would focus on the ageing of the brain and abnormal cell proliferation.

Launching the agenda, committee chairman Julius Krevans, from the University of California at San Francisco, said the goal of ageing research should be to combat disabling diseases that afflict older people rather than to extend life *per se*. But mindful of the present drive to control public spending, Krevans also argued that the programme could save money.

The over-65 population of the United States is forecast to grow from about 31 million to almost 40 million over the next 20 years, and the cost of health care for the old is forecast to double over the same period.

Krevans said that if the programme could delay the time that old people are admitted to nursing homes by an average of just one month, it could save several thousand million dollars each year.

Peter Aldhous

## MEDICAL RESEARCH

# Beating the tissue shortage

## Washington

AN UGLY dispute over the handling of human brains from the Philadelphia coroner's office is spotlighting a nationwide shortage of brain tissue for medical research.

Philadelphians reacted with outrage last week when media reports revealed that the Philadelphia Medical Examiner had removed the brains from about 25 cadavers and sent them to the University of Pennsylvania Medical School, where most were used for classroom instruction. The problem was that the transfers had been undertaken without the knowledge or permission of the families of the deceased.

Officials defend the transaction as completely legal and of "mutual benefit". The medical school got fresh brains from the cadavers of people who had been previously healthy, and the coroner got free dissection reports on possible victims of foul play by the medical school pathologists who examined the brains with their students.

The families of the deceased were not persuaded by these arguments, however, and the coroner's office has temporarily stopped the brain trade while it considers its options.

The medical school's arrangement with the Medical Examiner offered a way to deal with a problem that bedevils schools across the country: a lack of human organs and tissue needed for research and for teaching. In particular, by helping the coroner's office, the medical school got to examine the brains

of generally young, healthy people — an unparalleled opportunity to spot early signs of neurological disease, as well to as collect postmortem epidemiological data on a cross-section of the population, not just the sick and elderly.

Collaboration between coroners and medical schools is common, says Philadelphia deputy medical examiner Ian Hood. In small cities, the medical examiner's office is often located in the local medical school hospital, and students are routinely invited to participate in autopsies. Hood believes that the only distinction in the Philadelphia case is that the entire brains were taken out of the building to be examined elsewhere, even if it was just a block away.

Observers of the brain deal disagree as to whether it is legal under state and local laws, and the ethics of it are just as problematic. Across the state from Philadelphia, former Pittsburgh coroner Cyril Wecht has his qualms.

"Technically, it really is professionally unethical," says Wecht, who is a University of Pittsburgh pathologist, a lawyer and a past president of the American Academy of Forensic Sciences. "I realize that gathering the appropriate permissions would be a procedural mess, but I believe that a substantial percentage of the families would be disturbed to discover that the brains [of their relatives] had not been buried with the rest of the body." If lawsuits force the brain deals to

stop, Hood says, promising research could suffer. "One of the saddest aspects of this whole thing is that we may have to curtail our research programmes", he says. The Penn researchers were looking for undetected lesions in the brains that might provide scarce data on the early, asymptomatic, progression of Alzheimer's and Huntington's disease. Hood's office was also collaborating with researchers at the Philadelphia Children's Hospital to study the bodies of apparent victims of sudden infant death syndrome, looking for the clues of inborn errors of metabolism.

**Christopher Anderson**

## ENDANGERED SPECIES

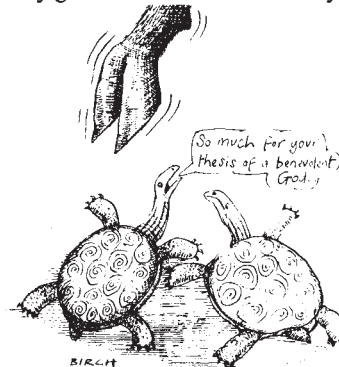
# Killer cows prompt shell suit

## Washington

ONE day last month, on a barren stretch of Arizona desert near the Utah border, a cow squashed a tortoise. The cow's forefoot sank 14 inches through loose sand, finally stopping in the underground burrow of a desert tortoise, who was home. After crushing the animal's shell, the cow pulled its foot out of the hole and walked on.

That would have been that if this were an ordinary tortoise. But it was not, and now the US government is about to be sued to prevent further such attacks by cows.

As one of fewer than 175 of the endangered desert tortoises in the Arizona-Utah desert, the doomed animal was wearing a radio transmitter so that it could be monitored by government researchers trying to



save the 10- to 15-inch species, which can live to be more than 100 years old. Within hours of the accident the scientists were on the scene, in time to photograph the cow tracks and euthanize the tortoise, which was still alive.

Last week, four environmental groups filed an intent to sue the US Department of the Interior and its Bureau of Land Management for allowing cattle to graze in the desert tortoise habitat. The Natural Resource Defense Council, Defenders of Wildlife, the Environmental Defense Fund and the Humane Society claim that the injuries suffered by the tortoise correspond closely to shell damage in other killings that had previously been attributed to coyotes.

**Christopher Anderson**

## SCIENTIFIC MISCONDUCT

# NIH publish new rules

## Washington

As expected, the National Institutes of Health last week published new proposed rules for the controversial Office of Scientific Integrity (OSI), whose existing rules were declared illegal by a Wisconsin court late last year. OSI hopes to legitimize its misconduct investigations by opening its procedures to public comment, as required by federal law (see *Nature* 349, 357; 31 January; 350, 100; 14 March 1991).

The way that OSI is going about it has drawn a number of complaints. Critics note that OSI has published the rules not in the 'Proposed Rules' section of the June 13 *Federal Register*, but in the 'Notices' section. The signature at the bottom of the proposal is not that of Health and Human Service secretary Louis Sullivan, but of his assistant, James Mason. To close observers these features are telltale signs that OSI neglected to get White House approval for the new rules.

Lyle Bivens, director of the Office of Scientific Integrity Review, which oversees OSI, confirms that the rules were not approved by the Administration. The reason, he says, is that the proposed regulations are not a "rule-making" but rather a "policy-

making", which has a 60-day period for public comment but needs no review from the Administration. "Our lawyers felt that the important message [of the Wisconsin ruling] was to get this [proposed policy] out for comment," not to actually go through the full rule-making process, Bivens says.

This is, one lawyer says, a "remarkably disingenuous reading of the ruling." Because the NIH rules serve as a model for similar misconduct procedures at other federal science agencies, the rules should have been developed with input from them, he argues.

Although NIH will continue the process of legalizing its new misconduct regulations, it is at the same time appealing against the Wisconsin decision. In the original suit, which charged primarily that OSI investigation violated the accused's rights to 'due process', NIH won on all major points except for the unexpected one of the office's legality. To make sure that the due process argument stays dead, last week's proposed rules include a new clause: "No opportunity is provided for subjects to confront and cross-examine other witnesses interviewed by OSI." Or, as one lawyer puts it, "no due process, no how."

**Christopher Anderson**



# The UK role in conservation

## London

WHAT role can the UK play in world conservation efforts? That was the question addressed last week at a seminar at the Natural History Museum in London, convened by the UK Department of the Environment (DoE).

The answer: Two centuries of British interest in natural history have produced in the UK an institutionalized body of expertise and unmatched reference collections that now constitute a vital global resource. UK scientists should exploit these capabilities.

"Generations of clergymen attending to their six-legged flocks" have made the UK biota "the best-studied anywhere", said Robert M. May of the University of Oxford. But this heritage obliges the UK to foster its own systematic biology research, which has been "seriously undermined", according to Alan Hamilton of the World Wide Fund for Nature. This is currently the subject of wide-ranging reviews by the Science and Technology Committee of the House of Lords, the Natural Environment Research Council and other organizations.

Nevertheless, many UK institutions have well-established links with developing countries. The Natural History Museum, for example, is working on longterm conservation projects in Costa Rica and Belize, whereas the Overseas Development Admin-

istration, much praised for its new report "Biological Diversity and Developing Countries: Issues and Options", has contributed £40 million to the new Global Environment Facility, a \$500-million fund jointly managed by the World Bank, the UN Environment Programme (UNEP) and the UN Development Programme.

The fund, which is designed to provide money for a variety of environmental programmes around the world, was hailed as a "major breakthrough" by Robin Pellew of the World Conservation Monitoring Centre. Nonetheless, it does have its teething problems. Vicente Sanchez, Chilean ambassador to Kenya and permanent representative to UNEP, said one stumbling block is the mistrust in which the fund is held by many in the developing world, who regard the World Bank with suspicion. Sir Crispin Tickell, Warden of Green College, Oxford, suggested that UNEP instead should be encouraged to take the lead in selecting suitable projects to be backed by the new fund.

The DoE seminar was convened to take up the challenge posed by former UK Environment minister Chris Patten in a speech last November, but its results will feed into the ongoing global consultation process that will culminate next June in the UN Conference on Environment and Development in Rio de Janeiro. DoE representatives will

use the seminar as a brief when they attend a UNEP meeting in Madrid this Monday (June 24) to put flesh on the bones of a framework convention that has already been drafted.

Further meetings are scheduled for September (in Nairobi) and January, and further consultations are certain before a workable convention emerges for signature at Rio.

The mood of the DoE meeting was one of urgency, as description followed graphic description of just how fast the world's natural genetic resources are vanishing, many of them to be lost forever no matter how hard people work to stem the tide. "We are simply committed to the disappearance of much of the world we know — that's a fact", May noted. This sobering thought has prompted serious research efforts into what Richard VaneWright, Chris Humphries and Paul Williams of the Natural History Museum have called the "agony of choice": deciding, on the basis of biodiversity measures, known systematic relationships and species richness, the areas of the world that must be given priority for saving on account of their inherent biodiversity.

Henry Gee

## REMOTE SENSING

### Satellite launch delay

#### Munich

PROBLEMS with the usually reliable Ariane 4 booster rocket have indefinitely delayed the launch of the satellite ERS-1, the first stage of the remote sensing programme of the European Space Agency.

The satellite was to have been launched on 3 May, but problems with the rocket prompted first a temporary and then a longer delay.

ERS-1 has now been removed from the top of the rocket and put into storage for at least several weeks. The earliest possible launch date is now sometime in July.

ERS-1 contains an ambitious package of remote-sensing instruments that will produce radar images of the Earth of an unprecedented scope (see *Nature* 351, 8; 1991).

The delay was caused by a pressure drop in one of the fuel lines in the upper stage of the rocket. The drop had been noticed on earlier launches, but only now has Ariane-space, the commercial launch company that provides the Ariane rocket, determined that it could lead to a catastrophic error if left uncorrected.

A spokesman for the space agency said the delay would not affect the quality of the ERS-1 observations, which can begin whenever the satellite is launched. But the delay caused much disappointment for the researchers involved, he added.

Other space agency science projects are not expected to be affected by the delay. The agency's next launch on Ariane is not due until the Infrared Space Observatory is launched in 1993.

Steven Dickman

## UK SCIENCE POLICY

# Tories respond to Labour plan

## London

"THIN gruel" was how one UK government minister described the opposition Labour Party's science proposals that were unveiled last week (see *Nature* 351, 512; 1991). The comment, from Trade and Industry Secretary Peter Lilley, was made as he launched a Conservative Party document, 'Science and Innovation: The Cultural Revolution'.

The Labour Party intends to fund an increase in research and development (R&D) funding from 1.8 per cent to 2.5 per cent of gross domestic product largely by offering tax breaks to corporate investors in R&D. In 'Science and Innovation', the government slams the Labour Party for proposing that "tax distortions and Whitehall civil servants would be better at bringing about innovation than the industrial innovators themselves". But the same document bemoans the persistent unwillingness of industrialists to invest in R&D despite 12 years of Conservative government and a plethora of schemes to encourage them to do so.

It is this lack of investment in industrial civil R&D that accounts for the decline of civilian R&D spending in the UK as a proportion of gross domestic product over the past 12 years, despite the fact that government investment in academically related

R&D is greater, as a proportion of gross domestic product, than it is in the United States and Japan (0.38 per cent, as against 0.31 per cent and 0.25 per cent respectively). The Labour Party does not question these figures — they are drawn from Table 1 of their own document, 'Pushing Back The Frontiers'.

The Labour document was cautiously welcomed by the Association of University Teachers and the pressure group Save British Science.

That group noted, however, that although Labour's intentions were "excellent", its "objectives remain largely unquantified". As for the proposal to create a science minister in the cabinet office, Save British Science continue to press for a science minister of full cabinet rank.

Speaking at Labour's press launch, Labour leader Neil Kinnock pointed out what he thought of as a telling spelling error in the Conservatives' document: it reads, "The Prime Minister already chairs a cabinet committee which overseas (*sic*) Science and Technology". Kinnock asked whether this might be a subliminal reference to the 'brain drain', the existence of which the government emphatically denies.

Henry Gee



# Frustration in Switzerland

## Zurich

As part of its 700-year anniversary celebration, Switzerland last month opened a huge and unique science exhibition on the outskirts of Zurich. Called Heureka after the German-language version of Archimedes' famous cry, the exhibition departs from the historical approach of many science museums and instead attempts to show the joys and anxieties of 'living science' by having researchers themselves show what they do to the public.

After the first month of the exhibition,

the state of the art. The result is that many of the 'experiments' allow visitors to do no more than push a button that causes some preordained, complex process to be carried out before their eyes. This is the case in displays on nuclear fusion and genetic engineering.

Furthermore, technical terms such as 'plasmid' are frequently used in the displays without definitions, leading many visitors to walk away in frustration.

More successful in the eyes of visitors are engineering projects like avalanche barriers



Eureka: Swiss birthday exhibition fails to displace the public's emotions

however, the encounter has left both sides feeling frustrated.

Researchers who contributed to the exhibition say they have been disappointed in just how uninterested people are in what they are doing. After five days of displaying a working laboratory model of a scanning tunnelling microscope, physicist Michele Bernasconi of the University of Basle says it is depressing how little people want to know.

Bernasconi says that "it took six months to build this display" of the microscope, "but I have only had a few questions a day. People seem afraid to ask me anything." Bernasconi's experience is typical of those reported by researchers at Heureka.

On the other hand, many visitors say they feel baffled by what the researchers are presenting. "We saw everything but understood very little," was the most common response of those interviewed.

The exhibits for the most part assumed too much background knowledge, said Christian Good, a visitor from Zurich, so that a person without a university education would generally be lost.

Biologist Isabelle Dusart of France agrees: "Without the training in physics that was required of me as a biologist, I wouldn't be able to understand what the physicists are presenting here."

The problems of Heureka show just how difficult it is to communicate about science to the public. Part of the problem is that the designers of Heureka tried to make every exhibit interactive and yet also reflective of

that can measure the weight of fallen snow or a futuristic tandem bicycle that allows visitors to test their pedalling efficiency.

That the two sides are somehow missing each other at Heureka is reflected in the low number of visitors who have so far attended — just 62,000 in the first four weeks, an average of about 2,000 people a day. In order to break even on the SF30 million (\$20 million) exhibition, says Heureka director Georg Müller, it will require 6,000 visitors per day throughout the summer months.

Müller, whose experience in directing a highly successful hands-on science exhibition called 'Phenomena' in Zurich in 1984 made him the prime candidate to put on Heureka, defends the premise of Heureka and says he is convinced that the exhibition will become more popular after a number of initial kinks have been worked out.

Ironically, the historical portion of the exhibition — housed in a rough wood spiral construct called Galileo's Tower, complete with internal staircase — has been a much bigger hit with the public than the current experiments. The tower features hands-on demonstrations of machines ranging from pulleys to telescopes, as well as demonstrations of basic chemistry that have proven very popular. It is, says one researcher who helped set up the exhibition, as if people's scientific intuition has reached only a point somewhere in the seventeenth and eighteenth century, and so they are only comfortable with experiments that date from that time.

**Steven Dickman**

## Japanese biologists produce 'hot' papers

### Tokyo

THE common perception that Japan is strong in engineering and applied science but weak in basic research is turned upside down in a recent survey of the 'hottest' research papers coming out of Japan by the Washington-based *Science Watch* journal.

In its recently released April issue, the journal's analysis of current 'hot' papers, defined as papers published between 1988 and 1990 that receive many more citations than average from other authors, reveals that Japan is strongest in basic research in biochemistry, molecular biology, and pharmacology, and weakest in engineering, technology and applied science.

Out of 42 hot papers from Japan in *Science Watch's* worldwide database of 900 hot papers, 25 were in the life sciences. This is considerably more than the 15 that would be expected based on the percentage of all Japanese papers in this field. In contrast, there is only one hot paper in engineering, technology and applied science, as opposed to the six expected.

The University of Tsukuba ranks highest among Japanese universities with 5 hot papers on endothelins — powerful peptides that raise blood pressure and reduce circulation. A string of recent papers by a group led by Tomoh Masaki at the university have stirred tremendous interest worldwide, partly because of the potential application of the research to treatment of heart disease, but also because the research is widely recognized as being a substantial contribution to understanding the fundamental biochemistry, molecular biology and pharmacology of these important peptides (see *Nature* 348, 673; 1990). Researchers at the University of Osaka also do well, with 5 top papers covering, among other things, research on interleukins.

A closer look at the ratings of scientific papers shows that the common perception about Japan's strength in applied science does have a basis in reality, however. When *Science Watch* looked at the average number of citations per paper for all papers regardless of their quality, Japan came out strongest in engineering, technology and applied science, with 11 per cent above the average worldwide citation score per paper. By the same measure, Japan was weakest in the life sciences with 26 per cent below the average number of citations.

It is only when the database is reduced to a limited number of hot papers with above-average scores that the life sciences come out on top. In short, the journal concludes that "technology reigns supreme when one considers Japanese research as a whole," but "life sciences top the chart when one looks at Japan's *creme de la creme*."

**David Swinbanks**

# Evaluating cancer therapies

## Washington

AFTER years of fighting with the insurance industry over the support of clinical trials, cancer researchers are moving to take matters into their own hands. Rather than continuing to bias their research towards those drugs and techniques that insurance companies will pay for patients to receive — neglecting promising therapies — members of the American Society of Clinical Oncology (ASCO) are planning to set up an independent evaluation panel in hopes of bringing insurance support in line with scientific consensus.

The panel, loosely modelled after the congressional Office of Technology Assessment, is expected to review both experimental and established drugs and techniques, evaluating what is known about their efficacy and listing appropriate circumstances for their use.

Researchers hope that such a panel can separate promising therapies from longshots — and weed out expensive treatments that are no longer the state of the art.

"We're looking at new and expensive technology," says Martin Abeloff, ASCO president and clinical director of the Johns Hopkins University Oncology Center in Baltimore. "But we're also hoping to show that the medical community can do some policing of itself, that it can abandon therapies it no longer considers useful." If so, it may help solve one of the principal problems in clinical cancer research: patient costs. Because most government research grants do not generally include money for patient care costs (on the assumption that insurance companies would have to pay for patients' care whether they are in or out of a clinical trial), researchers are dependent on the insurers to cover all but the direct research costs.

If insurance companies decide that the treatments are "experimental" or "investigational" rather than "generally accepted" as a treatment option, they often will not support them on the grounds that unproven and expensive therapies are usually not in the best interests of the patients. If an insurance company has \$150,000 to spend on a breast cancer patient's care, they ask, should that go to one experimental autologous bone marrow transplant (ABMT), or to a dozen less extreme — and better understood measures? In recent years, as insurance companies found their resources strained by rising health care costs, the default position has been to support no investigative therapies, except under pressure from the courts. Not surprisingly, that has made life difficult for cancer researchers, who have in some cases been forced to postpone trials on promising, but unconfirmed, drugs when their trial participants could not get insurance support.

For example, Larry Norton, chief of breast cancer residency at Memorial Sloan Kettering Hospital in New York, says his research

on taxol, an experimental compound extracted from the bark of the Pacific Yew tree, has been delayed because about half of the participants in his trials are refused insurance coverage. He is forced to recruit twice as many participants as he needs, a process that can take months.

But Norton, like many other researchers, does not blame the insurance companies for his troubles. "It's a complicated problem with no heroes and no villains," he says. The companies are usually trying to get the most effective care for their money, he points out. But when they are confronted with something like an ABMT, they are put in a quandary.

At something on the order of \$150,000 a treatment, ABMTs — in which a patient has her bone marrow removed, is given massive chemotherapy treatments, and then has the marrow returned — are tremendously expensive. For most breast cancer patients, an ABMT is unlikely to work better than chemotherapy alone. Even in the small percentage of patients where ABMTs are considered a good bet, no one knows how effective they are in eradicating cancer for good; few patients have been followed for more than a year or two.

Overall, researchers say, ABMTs are probably only effective in a few per cent of breast cancer cases.

When insurance companies decide not to support an ABMT for a patient, they usually cite experts who say that the treatment is still unproven and "investigational". Sometimes that is the end of the line for the patient. But, increasingly, patients are taking the companies to court and winning. Of 15 cases last year, 13 were decided in favour of the patient. Few judges want to deny a cancer victim any chance — no matter how slim — for recovery. If ABMTs really are an effective treatment, compared to other therapies, then this is all for the best. But if they are not as effective as some practitioners claim, and sap scarce treatment dollars that could be used in more promising trials, ABMTs could be slowing the progress of cancer research.

This is the sort of issue that ASCO hopes its panel can address. Abeloff says that ABMTs may be used as a trial balloon to gauge the ability of the panel to reach consensus and the response of the insurance industry.

So far, the industry seems enthusiastic. "We would very much encourage this," says James Cross, medical director for claim operations for the Travelers Companies, a major health insurance group. "It is very difficult for us as insurers to know what the state of the art really is." But he cautions that the panel must be careful not to stamp every new therapy with a seal of approval. "It will be very important for ASCO to do it well and to be very critical, to take a good look and not just a superficial examination. If they err on

the side of approving everything, it won't have the same validity." Travelers has set up assessment panels of its own, assembled from researchers from well regarded hospitals and research centres.

But such expert panels are no guarantee in court, where judges are confronted with often contradictory expert testimony. Were an independent group with the backing of a professional society like ASCO to decide that ABMTs are still "investigative" rather than accepted, courts might give it more weight.

Beyond ABMTs, candidate therapies for ASCO assessment include taxol and colony stimulating factor. The panel may also examine "off-label" drug uses — drugs that have Food and Drug Administration (FDA) approval for treating one disease, but may be useful for another disease, or in combination with other drugs. In all, about half of chemotherapy drugs have uses beyond those for which they were approved. But many insurers will not pay for any off-label use, even as part of an approved clinical trial. For instance, researchers have found that carboplatin (which was originally approved for ovarian cancer) may be even more useful in treating small cell lung cancer, but many insurance companies still refuse to pay for this use.

Next step for ASCO is a report this fall from an *ad hoc* committee that is studying how to set up such an assessment panel.

If all goes well, the panel could be up and running some time next year.

This week the issue will come before Congress, when Senator Brock Adams (Democrat, Washington) chairs a hearing on breast cancer therapies and insurance industry support problems.

**Christopher Anderson**

## SCIENTIFIC MISCONDUCT

### Conflict in NIH panel

#### Washington

CHARGING conflict of interest, the NIH have asked one of the members of the panel investigating Thereza Imanishi-Kari to resign.

University of Chicago immunologist Ursula Storb wrote a letter of recommendation for Imanishi-Kari in 1986, NIH found, but did not disclose that fact when she was asked to join the investigative panel. Storb was one of two panel members who wrote a dissenting opinion earlier this year when the NIH panel concluded that Imanishi-Kari had fabricated much of the data in a 1986 *Cell* paper. Storb told the *New York Times*, which reported the charge last week, that she had forgotten about the job recommendation. Even if she had remembered, she said, she would not have raised it because she did not consider such a relationship to be a conflict of interest. She has declined to resign. □



## European Community actions

SIR — In his article "Turmoil in European Biology" (*Nature* 351, 91; 1991), Lennart Philipson presents his views on how to strengthen basic biology in Europe, and I should like to add my comments.

■ The research and development activities of the European Communities (EC) are based on the Treaty (article 130f) which states that: "The Community's aim shall be to strengthen the scientific and technological basis of European industry and to encourage it to become more competitive at international level". This is the legal basis for all our programmes, including those containing elements of basic biology. Moreover, the EC must respect the 'subsidiary' principle, meaning that it acts only where it can add value to national or other actions.

The other organizations listed by Philipson are all perfectly respectable, and have their own aims and terms of reference, which differ from those of the EC. Others can be listed, such as the European Federation of Biotechnology, European Federation of Immunological Societies, and the European Union of Societies for Experimental Biology, which the EC helped create.

The EC mandate does not of course prevent us from developing both official and working relations with other organizations to our mutual benefit, or strengthening particular areas of research, such as plant molecular biology, which we considered insufficiently developed, even at the European Molecular Biology Laboratory.

■ There have been problems in the payment of grants, not only under the SCIENCE programme. They were the consequence of changes in budgetary procedure and have now been resolved. But to write that they were "precipitated by a commissioner who failed to receive support" is untrue. These problems had nothing to do with the Commission's proposal on "Human Capital and Mobility" and the SCIENCE programme will run for its appointed duration.

■ As regards genome projects, I think that EC actions on, for example, yeast, *Arabidopsis* or the human genome are seen as useful and well-managed by informed researchers and decision-makers, although the resources we devote to them are limited.

■ Our review process is certainly not perfect and, like any system, can be improved. Everybody knows, and surveys show, that peer review is preferred by most good scientists. What is the basis of Philipson's allegation that the EC "has a questionable peer review process and often distributes funds on geographical rather than scientific criteria"? Is it a matter of quality? Many of those who evaluated the BRIDGE (biotechnology) proposals had been fellows of the European Molecular Biology Organization. Is it representativeness? Our experts come both from academic institutions and industry. Is it confidentiality or anonymity? We go to

extremes to ensure both of these.

The EC does not use geographical rather than scientific criteria. There are first-class scientists in all member states, but our basic criterion of selection is scientific quality. Only then does the EC promote collaborations between countries and regions, between industry and agriculture, between industry and academic institutions, between users and producers, and so on.

■ Philipson seems to imply that the EC's usual mechanism is the "limited call for proposals". Our normal procedure is the "open call for proposals". Limited calls are exceptions, used only when we are certain that only one or very few laboratories are performing a precisely defined task.

■ As for the proposed remedy, it seems to me more grandiose than realistic. What should be frightening to Philipson is that it could be examined by science administrators who have "either failed in research or have been trained in law or economics", and that the "unqualified administrators" or the EC could still play a role in this process.

F. VAN HOECK

Director of Biology,

Commission of European Communities,  
Rue de la Loi 200,  
B-1049 Bruxelles, Belgium

SIR — Lennart Philipson, in characteristic fashion, pulls no punches when he boldly identifies a problem in most science administrators who influence decision-making — that they "either have failed in research or have been trained in law or economics, their loyalties resting entirely with the organizations or the ministry for which they work".

This is because biologists who fail to achieve tenure have no attractive option and administration is an alternative career. The problem could be reduced, however, if more doctoral students were recruited from the professional branches of biology — medicine, dentistry and veterinary science — which already have natural career paths in a return to clinical practice. The talented ones would stay in basic research.

The high earning power in law and accountancy would suggest that anyone from those fields seeking a career in administration is unsuitable by definition. Furthermore, the UK Charity Commission is unhappy if administrative and fund-raising costs exceed 5 per cent and this is a yardstick with which to cull the European science mandarins. Charles Dickens allowed Mr Micawber to outline to David Copperfield the principle of good financial management.

LARS H. BREIMER

Department of Chemical Pathology  
and Human Metabolism,  
Royal Free Hospital School of  
Medicine,  
London NW3 2PF, UK

## Advance from Australia?

SIR — As an expatriate Australian researcher, hoping for a future in my homeland, it is with great dismay that I see the Australian university system once again a subject of international concern (*Nature* 350, 447 & 452; 1991). It would appear that the Australian government sees fit to dismantle the Australian National University in the name of economic efficiency and a perceived political need to direct its research effort.

Unfortunately, Australia seems either economically unable or politically unwilling to allow the development of truly outstanding research-driven universities. It is true that there is a lack of academic tradition in the country, but with most universities in existence for less than a century, one cannot be surprised by this.

The Australian government's need to control and direct university research may stem from its inexperience. After all, were the structure of DNA, restriction-modification or DNA sequencing, the cornerstones of modern biology and medical research, discovered because of a government-imposed directive or decree? Independent enquiry, not strategic planning and bureaucratic intervention, underlies scientific discovery.

But in spite of the problems, university education in Australia has not been all bad. The number of Australian-educated scientists doing outstanding research in both the United States and Europe attests to this fact. Expatriate Australian scientists head departments in some of the world's best research institutes, universities and hospitals and are found on the editorial boards of leading international journals.

The problem with Australia seems to be not with the quality of education but the quality of its academic environment and quantity of academic opportunity. Many of the best leave the country after training because of the shortage of suitable employment or lack of tolerance and respect for academic achievement by the Australian population. Many who return after an extended stay in the United States or Europe find they are locked out of the system and, after pursuing successful careers overseas, cannot understand why Australians do not support academic excellence.

Academic research is part of the spectrum of human endeavour. If tolerated and given the opportunity, these endeavours can, and do, make important contributions to a country's economic, social and cultural well-being. Europeans and North Americans have learned this. Unfortunately, Australians have not.

JAN ROHOZINSKI

Department of Virology and Molecular Biology,  
St Jude Children's Research Hospital,  
332 North Lauderdale, PO Box 318,  
Memphis, Tennessee 38101, USA

# Darwinism not tautological

SIR — I would question both the ability and the need of Torres's work<sup>1</sup> to defend the theory of natural selection from the accusation of tautology<sup>2</sup>. Torres presents a novel example of an evolutionary optimization model<sup>3</sup>, in which the 'instantaneous fitness' of a set of phenotypic variants (in this case metabolic pathways) is defined according to a set of plausible rules (in this case thermodynamic criteria). All such models stand or fall on how well they predict the reality found in nature. If there is an anomaly, the model is modified *a posteriori*, by changing the criteria, by introducing constraints to the phenotype set or by adding criteria from other models (for example, sexual selection criteria that result in traits being far from thermodynamically efficient in terms of individual survival). Thus, it can be argued, calling a phenotype 'instantaneously fit' equates to saying that it is there in the first place, and so we are back to the original tautology: 'survival of the survivors'.

The tautology argument is nevertheless flawed because, as many evolutionists since Darwin have reiterated, natural selection emerges as an inevitable consequence of observed facts and empirically derived precepts. To deny the existence of natural selection, therefore, is to deny that organisms depend on limited resources for their survival, that they reproduce with heritable variation, and that this variation will affect their ability to exploit those resources. It is worth noting that Popper no longer considers natural selection to be tautological<sup>4</sup>, although he still considers it to be a "metaphysical research program" in that to assume natural selection accounts for *all* evolutionary history (to the exclusion of other mechanisms) is more or less unfalsifiable. So far, however, no alternative fact-based mechanism is known that also accounts for the exquisite adaptation of organisms to their environment.

MICHAEL E. WEALE

Department of Biology,  
University of Southampton,  
Bassett Crescent East,  
Southampton SO9 3TU, UK

1. Torres, J.-L. *Il Nuovo Cimento* **13D**, 177 (1991).
2. Maddox, J. *Nature* **350**, 653 (1991).
3. Parker, G. A. & Maynard Smith, J. *Nature* **348**, 27–33 (1990).
4. Popper, K. R. *Dialectica* **32**, 339–355 (1978).

SIR — John Maddox has raised once again the "nagging doubt" that the "theory of natural selection" is tautological. This is easily resolved once it is realized that natural selection is a mechanism, not a theory. In a system of replication with mutation, natural selection *must* occur. This is why Darwin called it "natural" selection, meaning "tautologically entailed" selection. The phrase "survival of the fittest" was introduced merely as an illustration for those who misunderstood the term natural selection, and is equally tauto-

logical. Attempts to avoid the tautology by redefining "fitness" can succeed only by making the phrase false as well as falsifiable.

This, however, does not imperil the scientific status of Darwin's theory of evolution using natural selection. Darwin claimed, first, that evolution had occurred and, second, that natural selection was the mechanism responsible. Both of these propositions are testable and falsifiable and therefore scientific.

COEL HELLIER

Mullard Space Science Laboratory,  
University College London,  
Holmbury St Mary, Dorking,  
Surrey RH5 6NT, UK

## Hinshelwood in the Pantheon?

SIR — James D. Watson's obituary of Luria<sup>1</sup> was a fitting tribute to a pioneer of molecular biology. There is no doubt that the Luria–Delbrück fluctuation test played a crucial role in establishing the spontaneous nature of mutations in bacteria, and made bacteria the organisms of choice for studying the nature of the gene.

However, contrary to Watson's remark, Cyril Hinshelwood did not argue against the mutational origin of resistant bacteria. Instead, he said there was another way they could become resistant, that is, through a change in their reaction pattern or chemical equilibrium. This he and his co-workers at Oxford established on the basis of the speed of change, the large fraction of the population involved, the finely graded nature of the response, the lack of precise specificity and the reversibility of the altered state<sup>2,3</sup>.

Unfortunately, the work of the Oxford group coincided with the beginnings of the remarkable development of molecular genetics, and it seemed irrelevant at the time, particularly because the changes could not be identified with any macromolecule, in contrast to the elegant simplicity of genetic change.

Closer inspection shows that the adaptive changes described by the Oxford group were analogous to the Dauermodifikationen of *Paramecium* described by Jollos and confirmed by Sonneborn<sup>4</sup>. Similar findings have since been made in plant<sup>5</sup> and animal cell culture<sup>6–8</sup>, and appear to be central to the processes of differentiation and neoplastic transformation. Indeed, the adaptive changes of large cell populations may well be the most common heritable changes they undergo, if we accept an inheritance that persists for many generations upon removal of the inducing constraint, but is subject to gradual reversal.

Thus, while molecular genetics flourished as a result of Luria's work, adaptive change languished, resulting in a one-sided

approach to the problems of development, ageing and cancer. I have no doubt that Hinshelwood will one day be placed on the same high level as Luria for his contribution to our understanding of the living state.

HARRY RUBIN

University of California, Berkeley,  
Department of Molecular  
and Cell Biology,  
229 Stanley Hall,  
Berkeley, California 94720, USA

1. Watson, J. D. *Nature* **350**, 113 (1991).
2. Dean, A. C. R. & Hinshelwood, C. *Nature* **199**, 7–11 (1963).
3. Dean, A. C. R. & Hinshelwood, C. *Growth, Function and Regulation in Bacterial Cells* (Oxford University Press, 1966).
4. Sonneborn, T. *Adv. Genet.* **1**, 263–358 (1947).
5. Meins, F. Jr & Binns, A. *Proc. natn. Acad. Sci. U.S.A.* **74**, 2928–2932 (1977).
6. Fox, M. & Radicic, M. *Mutat. Res.* **49**, 275–296 (1978).
7. Harris, M. *Cell*, **29**, 483–492 (1982).
8. Rubin, A. L., Yao, A. & Rubin, H. *Proc. natn. Acad. Sci. U.S.A.* **87**, 482–486 (1990).

## Novel reading

SIR — Steven Henikoff and Robert Levis (*Nature* **350**, 9; 1991) suggest that the use of the word 'novel' in the biomedical literature is growing dramatically. Although this seems indeed to be the case, they do not look into possible explanations for this increase. As 'novel' means 'of a new kind; different from anything seen or known before', its use is certainly justifiable on occasion, and may often be preferable to words such as 'unique', which presupposes that no other examples will subsequently be found, or 'brand new', which suggests that it did not exist before its discovery. 'Somewhat atypical' might often be more accurate, but with journals setting limits on title and abstract length, 'novel' has an edge in being only five characters rather than 17.

The increased use of 'novel' may also be exaggerated in certain fields. From January 1989 to March 1991, only 1.1 per cent of all Medline citations use the word 'novel', but 3.5 per cent of citations on HIV (human immunodeficiency virus) claim novelty. Although HIV papers for the same period make up 2.3 per cent of the citations, 7.2 per cent of the 'novel' papers are on HIV. Given that there are now over 60,000 papers on HIV catalogued in Medline (nearly seven papers per nucleotide pair), it is difficult to believe that another few hundred novel discoveries will be made each year. This brings up another question: if an organism needs only *n* nucleotide pairs completely to describe itself, then how many papers are required fully to understand the organism? Even the venerable bacteriophage T4 (which many people seem to think is 'solved') can claim only 1,226 citations (0.007 per nucleotide pair) in the entire Medline database since 1966.

ALAN C. CHRISTENSEN

Department of Biochemistry and  
Molecular Biology,  
Thomas Jefferson University,  
Philadelphia, Pennsylvania 19107, USA



# Making atoms march in step

The use of optical molasses to make atoms cool may now be familiar, by adding onto that a process of velocity-selection seems to have produced the coldest atoms yet.

THERE seem no bounds to the ambitions of those who work with atoms held in one or other of the various types of electromagnetic traps now on the intellectual market. For much of the past half a dozen years or so, the objective has been cooling: how best to massage atoms with finely tuned lasers so as to rob them of all but the last remnant of kinetic energy. People now boast of atoms with so little kinetic energy, or momentum, that their effective temperature is only a small fraction, perhaps a few millionths, of a degree Kelvin. Now the same small community's interest seems to be reaching out for even more ambitious goals.

But first, the technique, for which Ashkin of the AT&T Bell Laboratories seems to have coined the beguiling term "optical molasses". The basic physics is simple. Irradiate an atom with laser light tuned to an absorption frequency and, whenever it absorbs a photon, it will acquire extra momentum in the direction of the beam of  $h\nu/c$  (where  $h$  is Planck's constant and  $c$  is the velocity of light). But then the atoms will re-radiate the photon, but in a random direction, so that it will on the average acquire extra velocity in the direction of the beam. There is no surprise in that.

If the objective is to keep an atom fixed in space, a more intricate arrangement is plainly necessary. It will not suffice that two counter-propagating lasers should impinge on the hapless atom. For if each were exactly tuned to the resonant frequency, the opposing forces would exactly cancel each other. And the more intense the beams, the greater would be the rate of the re-emission of photons and, therefore, the more rapid the drift of the untrapped atom transverse to the directions of the beams.

The trick in making molasses is thus to tune a laser to a frequency just below that of the resonance line, but still somewhere near it. Then (on the average) the force on a single atom will be unsymmetrical. If the atom should be moving against the direction of the light, it will "see" blue-shifted light and will become a more efficient absorber thereof; if moving in the other direction, however, it will see red-shifted light, even further from the resonant peak.

So, on the average, the force on an atom will be determined by the direction of its motion; it is accelerated if moving in the direction of the laser beam, but much more sharply decelerated if moving in the opposite direction. But those are just the circumstances in which two counterpropagating laser beams tuned to just below the resonant fre-

quency will rob an atom of velocity, at least in the direction defined by the counter-propagating beams.

So why not have three pairs of counterpropagating beams, at right angles to each other, when the viscous forces of optical origin would seem to be the same in all directions? That, in essence, is the principle of optical molasses. Atoms do indeed travel more slowly when subjected to the treatment, but — as always — there are limits, for which the yardstick is the recoil momentum acquired by an atom absorbing a single quantum of radiation and the theoretical limit is the square of that divided by twice the atomic mass. The record so far is for an effective temperature of 2.5  $\mu$ K for caesium atoms.

It is important that optical molasses are not means by which atoms can be permanently trapped. They are simply devices by which the diffusion of an atom in a vacuum can be inhibited. Optical molasses rob atoms of velocity, and thus "cool" them, and even inhibit diffusional drift.

So what is new? This. A group of people at the Physics Department at Stanford University including Steven Chu (an associate of Ashkin's at the AT&T Bell Laboratories in the mid-1980s) have used an assemblage of sodium atoms cooled by optical molasses to create what they call a "fountain" — a stream of upward travelling atoms extracted from a bed of optical molasses which hardly differ from each other at all in their velocity (Kasevich, M., *et al. Phys. Rev. Lett.* **66**, 2297; 1991).

Sodium atoms are natural candidates for the tricks described because of the hyperfine splitting of the state responsible for the well-known sodium doublet in the optical spectrum. One well-known trick is to use optical pumping to fill one of these states with an assembly of sodium atoms which is then irradiated by counterpropagating laser beams tuned to each of the two frequencies. Then a kind of Raman (or "beats") effect populates the unfilled state with exquisitely cooled atoms. Velocities of 30  $\mu$ m a second are reported, which amounts to saying that a typical sodium atom will travel about one metre in an hour. Tortoises, despite their reputation, can do better.

How to extract upward travelling sodium atoms from such a device? It is simply a matter of changing the frequencies of the two lasers controlling vertical movement of the atoms. The frequency of the downward-propagating beam is decreased, and that of the upward-propagating beam increased (by the same amount of 2.9 MHz) for rather less

than a millisecond. Then, the authors say, they had an upward-moving beam of atoms in ballistic flight at 250 cm a second.

How to cool them further? The next trick is to discard from the beam atoms moving at velocities other than those determined by polarized light from two lasers differing in frequency by the difference between the hyperfine lines of sodium. This is the velocity-selection process crucial to this latest application of Raman cooling. The authors reckon that the velocity dispersion in their beam is that one would find in a static assemblage of sodium atoms at 35  $\mu$ K, and that they are able to use Raman interference to select a velocity-slice from that distribution that contains only one in 10,000 of the original population.

The demonstration of that is the most arresting feature of an article already sufficiently arresting: how do you measure the properties of a stream of sodium atoms ejected upwards from a bed of optical molasses in the course of less than a millisecond? Ballistically, of course. First, you make a baffle with a small 1.6 mm hole in it, and you worry only about the atoms that pass through it. You need an optical device for ionizing the atoms that protrude above it, so as to make them measurable, then you measure where they are at different times.

There seems little doubt that ballistic flight is the root cause of what happens; the article shows two distributions for the positions of sodium atoms after 140 milliseconds and 370 milliseconds, the former on the way up and the latter on the way down. The two distributions are displaced by about 3 mm, which is attributed to a departure of the track of the Raman beams from the horizontal.

Luckily, all this is not just fun and games. First, there are obvious applications in research. The authors reckon that the velocity spread in their upward moving beams is equivalent to 24 picodegrees Kelvin — much less than anything else achieved so far. The obvious possibility suggests itself that these same atoms might be trapped, near the top of their ballistic range, by electromagnetic devices of the now familiar kind.

There is also an interesting conundrum to be tackled by those with a stomach for these ventures. One could imagine a cascade of velocity selection steps each designed to fill another bed of optical molasses with neutral atoms of some kind. The end result could be a collection of atoms whose velocity distribution is much narrower than anything allowed for by Maxwell's distribution. That, at least, is what the authors say.

**John Maddox**

# Cagey carbon conundrums

Warren E. Pickett

ALTHOUGH the soccerball-shaped buckminsterfullerene molecule  $C_{60}$  has only recently been prepared in large quantities, already the solid has presented a number of surprises. When intercalated with alkali metal atoms to form the 'fullerides'  $A_xC_{60}$ , the material becomes superconducting below temperatures  $T_c$  of around 18 K and 28 K for  $A=K$  (ref. 1) and  $A=Rb$  (ref. 2), respectively. On page 632 of this issue<sup>3</sup>, Stephens *et al.* describe the crystal structure of single-phase, stoichiometric  $K_3C_{60}$  and show that there is true bulk superconductivity (at  $T_c=19.3$  K) in this compound. It should now become possible to look for the mechanism of superconductivity in the fullerides.

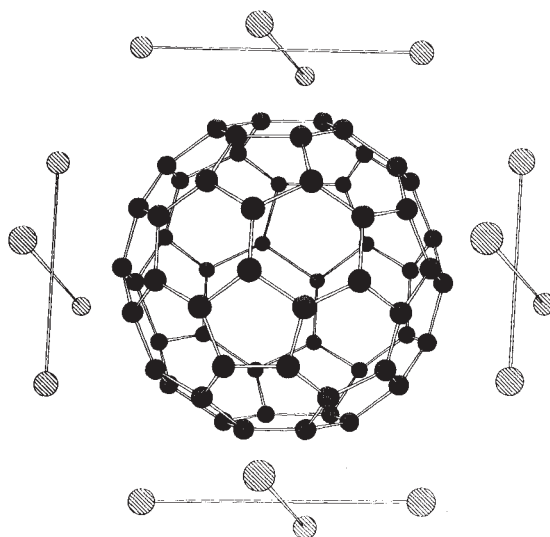
For many purposes of solid-state chemistry and physics, the  $C_{60}$  molecule is a single atom-like entity. For example, its shape and size seem to be virtually identical<sup>3</sup> in the vapour phase, in solid  $C_{60}$  and in solid  $K_3C_{60}$ . In this spirit it seems fair to endow it with the 'elemental' symbol Bf (buckminsterfullerene) so that, for example,  $Rb_xC_{60}$  becomes  $Rb_xBf$ . The Bf cage may in fact behave much like an atom with properties that are beyond the range of the usual atoms.

The fullerides have been heralded as the first examples of three-dimensional organic superconductors, which would be important not only because of the relatively high value of  $T_c$  but also because three-dimensional materials are usually easier to understand and to exploit. Most conventional superconductors are three-dimensional, effectively cubic if not precisely so. The high- $T_c$  copper oxides ( $T_c$  up to 125 K) are highly anisotropic, quasi-two-dimensional materials, and the previously known organic superconductors are quasi-one-dimensional. The fullerides are clearly three-dimensional materials (cubic), yet there is the intriguing possibility that the large Bf cage (roughly 10 Å in diameter) may possess crucial 'zero-dimensional' (atomic cluster) characteristics that make this a new class of superconductors.

The body of accepted knowledge of these fullerides is growing rapidly. The Bf solid is a weakly bonded material; but when it is intercalated with alkali atoms, electrons are transferred to the Bf cage, resulting in an ionically bonded solid. There have been indications that Bf has the ability to accept several electrons. New calculations<sup>4</sup> establish that in the fully intercalated, insulating  $K_6Bf$  (see figure) compound the Bf cage indeed assumes the highly ionic configuration  $Bf^{6-}$ . This high charge state, which may be stable only in the crystal field of the ionic solid, is indicative of a highly polarizable ion reminiscent of the oxygen ions that are effectively  $O^{2-}$  in the insulating copper oxides, but  $Bf^{6-}$  is probably much more responsive to disturbances. In the superconducting copper oxides, the

oxygen ion is open shell (not fully doubly charged) and an analogous open-shell situation (triply charged, on average, out of the possible six extra electrons) apparently occurs in the superconducting fullerides.

Superconductivity results from a pairing of electrons and a coalescing of the pairs into a macroscopic quantum state that is relative-



Arrangement of the potassium atoms (emphasized with crosses) around the  $C_{60}$  cage in the  $K_6C_{60}$  crystal. The potassium atoms at the front and back are omitted for clarity. (Courtesy of M.R. Pederson.)

ly robust; for example, it conducts current without scattering from impurities. The mechanism of pairing in the fullerides will surely be widely discussed in the near future. So far, experiment has ruled out none of the many possibilities. One discussion<sup>5</sup> has assumed the pairing of electrons by quantized atomic vibrations (phonons), which is the mechanism in conventional superconductors. The phonons in  $K_3Bf$ , although probably characteristic of those in cluster compounds, will be highly unusual for a superconductor. Preliminary calculations (R. Jones, personal communication) indicate that the internal Bf vibrations extend up to the high frequency of  $1,600\text{ cm}^{-1}$  (2,300 K in temperature units), which should survive relatively intact in the solid. In the fulleride crystals there will be very low-energy acoustic vibrations, owing to the superatom's mass of  $60 \times 12 = 720$  atomic mass units (just over thrice that of the heavy atom uranium, 238 a.m.u.). Finally, there will be optical modes involving the alkali ions, at some as yet unknown intermediate energy. Although the conducting electrons do not reside on the alkali ions, these ions will couple to the superconducting carriers on the Bf cages through electrostatic interactions, just as occurs in the copper oxides<sup>6</sup>. The relative (not to mention the absolute) strength of coupling of electrons to each of these types of

vibration is difficult to guess.

Because of the anticipated high polarizability of the  $Bf^{3-}$  ion in  $A_3Bf$ , Rosseinsky *et al.*<sup>2</sup> have suggested that the coupling could be purely electronic ('excitonic') in nature. For this to happen the direct Coulomb repulsion between two electrons must be over-screened in some way to give rise to an effective attraction. Another possibility involves a cooperative electron-cage effect: adding an extra electron in the available antibonding state of Bf adversely affects the cage's geometry enough for it to become

favourable for yet another electron to share that cage, resulting in local pairing. From this viewpoint the Bf 'atom' has extra degrees of freedom not present in real atoms. This 'polaron/bipolaron' picture is made more plausible perhaps by noting the analogy with boron cluster compounds — boron is next to carbon in the periodic table, and its clusters also form large molecular cages. These boron compounds are understood as polaronic materials that distort as charges move; nevertheless, there are also many dissimilarities between the borides and the fullerides.

Reportedly, the fullerides conduct only poorly above  $T_c$ . Whether this is intrinsic or is attributable merely to poor sample quality is not yet clear. The consensus being that the superconducting electrons reside

on the Bf cage, the character of the normal state conductivity is a crucial question. What are the important features for describing electrons jumping from Bf to neighbouring Bf? Is the transfer purely band-like, the molecular states merging to form a broad range of energy states that extends throughout the material, with hopping determined solely by overlapping wavefunctions on neighbouring Bfs? Or are correlation effects important, so that one must consider how electrons best avoid each other as they move through the crystal? The most standard source for models of electron correlation, on-site Coulomb repulsion, will be rather small owing to the large size of Bf, and electrons may prefer to hop freely and ignore this small repulsion. Dynamic correlation on a single Bf cage may be more important: a second electron on a Bf unit can minimize its repulsion with the first one by arranging to spend as much time as possible on the opposite side of the cage (all the while both are in fact spread over the entire molecule).

A simple way for two electrons to keep out of each other's way is to have aligned spins; then quantum-mechanical exchange keeps them apart. For three electrons per Bf, which is the case for superconducting  $K_3Bf$ , this argument suggests a Bf 'Hund's rule' analogous to the corresponding law for atoms, which states that atoms with unfilled shells



# Between B cells and T cells

Anthony L. DeFranco

arrange their electrons so as to maximize their total spin to lower their energy. Such arguments may be applied to suggest a spin-based mechanism of pairing for the superconductivity. However, this spin alignment will be opposed and perhaps overcome by crystal field effects and electron hopping, which split and broaden the energy levels. Indeed the accompanying magnetic behaviour that such spin-aligned electrons should display has not been seen.

The simplest band viewpoint of these solids systematizes some data nicely but appears to be contradicted by other data. The neutral Bf cage has a sixfold-degenerate energy level available for accepting electrons, and a substantial electron affinity that makes the Bf cage attractive for electrons. The Bf solid ( $K_0Bf$ ) is an insulator,  $K_3Bf$  is a metal (and, below  $T_c$ , a superconductor), and  $K_6Bf$  is insulating. This is consistent with the simplest rigid-band picture, which predicts  $K_3Bf$  to be a half-filled-band metal, whereas the  $x=0$  and  $x=6$  compounds are filled-band insulators. Photoemission data<sup>5,7</sup> suggest, in contrast, that upon adding only some K atoms, and hence some electrons, an entire band about 1 eV wide is fractionally occupied, whereas the 'rigid-band' picture would imply that a lower fraction of the band would be fully occupied. This is potentially an important hint into the nature of the electronic structure, indicating Bf charge states that vary in time or space, or it may be due simply to inhomogeneities arising from local differences in K concentration that would broaden the spectrum<sup>5</sup>. However, keeping in mind that photoemission is exceedingly sensitive to surface properties, further experiments are called for.

We have the picture, then, of super-heavy, super-polarizable Bf ions with extra internal degrees of freedom forming a solid with interspersed alkali ions, giving rise to rather high-temperature superconductivity. The one clue regarding the mechanism for pairing is that pressure strongly decreases<sup>8</sup>  $T_c$  whereas replacing K with the larger Rb ion (negative chemical pressure) increases  $T_c$  from 18 K to 28 K. Thus in this range of densities it is more favourable for superconductivity for the Bf clusters to be further apart. But it seems that at present nothing can be ruled out, nor ruled in.

With the demonstration<sup>3</sup> that macroscopic, single phase, stoichiometric samples can be prepared, the way is opened for the wide variety of experiments that are needed to rule out some of the possible mechanisms and narrow the search. □

Warren E. Pickett is at the Naval Research Laboratory, Washington DC 20375, USA.

A CENTRAL event in the immune response is the antigen-specific interaction between a helper T lymphocyte and a B lymphocyte, leading to their mutual activation. Molecular studies defining the cell-surface proteins involved in this cellular dialogue have revealed how rich and complex it is. Now, on page 662 of this issue<sup>1</sup>, Van de Velde *et al.* show that two of the earliest known lymphocyte cell-surface molecules, Ly1 (CD5) and Lyb2 (CD72), are involved in the process as a pair of interacting receptors.

The antibody response to most protein antigens requires that the relevant B cell must recognize antigen with its membrane immunoglobulin receptor molecules, and that it must receive certain activation signals from a helper T cell; such signals can include both secreted interleukins and factors resulting from cell-cell contact. For some types of antigens, such as the erythrocyte-based antigens, growth and differentiation factors secreted by the T cell provide the required activation signals. Helper T-cell recognition of antigenic peptides bound to class II molecules of the major histocompatibility complex (MHC) leads to secretion of these interleukins. Secretion can be directed towards the site of contact with the B cell<sup>2</sup>. For many other antigens, such as soluble or aggregated proteins, no combination of known interleukins can replace contact with the helper T cell, suggesting that interaction of molecules on the surface of the helper T cell and the B cell provides a signal to the B cell that promotes its activation. In support of that view, membranes from activated T cells have been found to cooperate with interleukins in promoting such activation<sup>3-5</sup>.

A number of interacting pairs of molecules have been identified that could send and receive cell-contact signals. Among them are the T-cell antigen receptor and the class II MHC molecule on the B cell; the adhesion molecule pairs LFA-1 and ICAM-1, and CD2 and LFA-3; and two newly described ligand/receptor pairs, CD28 and B7, and CD5 (Ly1 in the mouse) and CD72 (Lyb2 in the mouse) (see figure).

The interaction of the T-cell antigen receptor with the peptide/MHC II complex sends a critical signal to the T cell, and perhaps also to the B cell. Antibodies against class II MHC molecules can induce proliferation of B cells that have been preactivated with interleukin 4 and anti-immunoglobulin (used as a surrogate for antigen)<sup>6,7</sup>. But in experiments where membranes from activated helper T cells stimulated B cells, expression of the appropriate antigen/MHC class II complex by the B cells was not required for stimulation. So another pair of interacting molecules was thought to be responsible for the signal inferred from these experiments.

An attractive candidate for the molecule receiving the activation signal is Lyb2/CD72 on the B cell, which is now shown to interact with Ly1/CD5 on the helper T cell. Van de Velde *et al.*<sup>1</sup> purified the CD5 molecule from a human T-cell line by antibody-affinity methods, tagged it with biotin and found that it would bind specifically and exclusively to B cells. To identify the ligand on the B cell that binds CD5, they examined the ability of a panel of antibodies against B-cell surface molecules to interfere with this interaction. Only anti-CD72 monoclonal antibodies blocked binding.

The possibility that CD5 interacts directly with CD72 is strongly supported by the authors' observation that purified CD5 binds to non-B cells (mouse L cells and human Jurkat

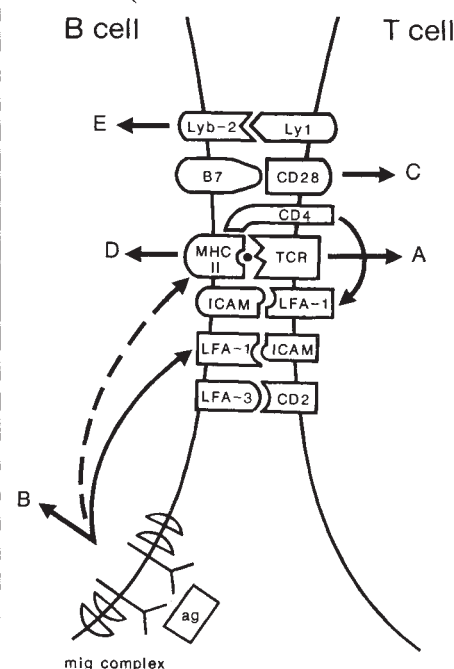


Diagram of the pairs of cell-surface molecules involved in the interactions between B cells and helper T cells. The dashed arrow represents membrane immunoglobulin (mig)-directed delivery of antigen (ag) to an intracellular compartment where it is degraded and peptides can combine with MHC class II molecules. Solid arrows show the discrete signal-transduction events that have been established. A and B are the antigen-receptor signal-transduction events involving tyrosine phosphorylation and phosphoinositide breakdown. The antigen receptors also regulate LFA-1 affinity for ICAM-1, possibly through the signal transduction events. In the T cell, CD28 also sends a unique signal to the T cell (C). In the B cell, class II MHC molecules and Lyb2/CD72 appear to induce distinct signalling events (D and E). Not shown is the exchange of soluble interleukins and binding to the corresponding receptors on the other cell. TCR, T-cell receptor; LFA, lymphocyte function-related antigens; ICAM, intercellular cell-adhesion molecules.

1. Hebard, A. F. *et al. Nature* **350**, 600-601 (1991).
2. Rosseinsky, M. J. *et al. Phys. Rev. Lett.* **66**, 2830-2831 (1991).
3. Stephens, P. W. *et al. Nature* **351**, 632-634 (1991).
4. Erwin, S. C. & Pederson, M. R. *Phys. Rev. Lett.* (submitted).
5. Benning, P. J. *et al. Science* **252**, 1417-1419 (1991).
6. Cohen, R. E., Pickett, W. E. & Krakauer, H. *Phys. Rev. Lett.* **64**, 2575-2578 (1990).
7. Wertheim, G. K. *et al. Science* **252**, 1419-1421 (1991).
8. Spam, G. *et al. Science* (submitted).

T cells) expressing transfected CD72 or the mouse homologue Lyb2 (ref. 8), but not to control cells. So it seems quite likely that Ly1/CD5 and Lyb2/CD72 are a pair of interacting molecules. As anti-Lyb2 antibodies can promote vigorous B-cell proliferation<sup>9</sup>, and can block T cell-dependent B-cell activation<sup>10</sup>, the possibility that this interaction has functional consequences for the B cell clearly warrants testing.

It is perhaps more likely that each pair of interacting cell-surface molecules will play an important role in collaboration between T cells and B cells. Given their known properties, communication between LFA-1 and ICAM-1, and CD2 and LFA-3, is probably required for cell-cell adhesion<sup>11</sup>. In the case of LFA-1 and ICAM-1, both molecules are expressed on both cells, permitting a bidirectional interaction; moreover, activation of antigen receptors on the T cell<sup>11</sup> or the B cell<sup>12</sup> causes LFA-1 to bind its ligand with higher affinity, an 'inside-out' signalling that is thought to regulate the binding of the cells to each other.

In addition, another pair of molecules on B cells and T cells, B7 and CD28, have recently been found to interact<sup>13</sup>. As antibodies to CD28 on the T cell act in synergy with anti-T cell receptor antibodies to promote interleukin 2 transcription<sup>14</sup> and stability of messenger RNA<sup>15</sup>, it is attractive to think that this pair of molecules sends a key signal to the T cell. This signal may be equivalent to the 'co-stimulatory' activity of antigen-presenting cells that has been shown *in vitro* to be required for T cells to make a strong positive response to antigen stimulation<sup>16–18</sup>. Indeed, a B7-IgG chimaeric protein provides the necessary co-stimulus for T-cell proliferation and accumulation of interleukin 2 mRNA<sup>13</sup>. Thus, the interaction of B7 on the B-cell surface with CD28 on the T-cell sur-

face is expected to send an important activation signal to the T cell that acts in synergy with the signal sent by the T-cell antigen receptor. Does this interaction cause B7 to deliver a signal to the B cell as well? The same question of bidirectionality can be asked in the case of the Ly1/CD5 interaction with Lyb2/CD72. Antibodies against Ly1/CD5 can stimulate T cells<sup>19</sup>, so this interaction may be sensed by both cells.

Finally, if several molecules are sending and receiving signals during communication between B and T cells, to what extent are the CELL CYCLE

signals distinct and to what extent are they separately regulated? Those questions remain to be answered, but the overall message from the recent string of results is that we are well on the way to a detailed understanding of the molecular events that trigger the immune response. □

Anthony L. DeFranco is in the Departments of Microbiology and Immunology, and Biochemistry and Biophysics, and in the G. W. Hooper Foundation, University of California, San Francisco, California 94143-0552, USA.

## Starting and stopping

Geoffrey North

CANCER is more than anything a disease of the cell cycle, but despite the advances made in identifying cellular components deranged in cancer cells, and those controlling the cell cycle, little is known about how the two are related. The signs from this year's Cold Spring Harbor Symposium\* are that this may soon change, with the discovery that a mouse growth factor can induce expression of a set of cyclins, known as cell-cycle control proteins, one of which is almost certainly the homologue of a human gene implicated in several types of cancer<sup>1,2</sup>. The new cyclins are expressed in the G1 phase of the cell cycle, between mitosis (or M phase) and S phase, when DNA is replicated, and it is in G1 that the crucial decisions are usually made that commit cells to division. Most rapid progress in understanding the key events of G1 is being made in studies of the budding yeast *Saccharomyces cerevisiae*. They have led to the discovery of a positive feedback loop involving the G1 cyclins, which is likely to be important in determining cell-cycle timing as well as being the point at which extrinsic signals intercede to halt the cell cycle.

### Start control

The cell cycle involves an ordered sequence of events (see Fig. 1), which is controlled at least in part by a specialized set of biochemical timing devices. A seminal discovery, made in 1988, was that homologues of the fission yeast cell-cycle control gene *cdc2* encode the catalytic subunit of maturation promoting factor (MPF), which can induce the transition between G2 and M phases (see ref. 3 for review). The catalytic activity of this subunit, a phosphoprotein termed p34<sup>cdc2</sup>, depends on its interaction with one of the cyclins, proteins that were first identified through the 'sawtooth' variation in their concentration in cells of marine invertebrate embryos.

The *cdc2* gene first came to light in fission yeast by the observation of the way mutations in it block the transition between G2 and M phase. Mutations in its budding yeast

homologue, *CDC28*, were however originally found to arrest the cell cycle in G1, at a point known as Start. It is now known that *cdc2* in fission yeast and *CDC28* in budding yeast are both required in G1 for transition to S phase, and in G2 for transition to M phase.

Start is, however, a particularly important control point in the cell cycle of budding yeast. It is the point at which the mating pheromones produced by the  $\alpha$  and a haploid cells ( $\alpha$ -factor and a-factor respectively) cause cell-cycle arrest, allowing cells of complementary mating type to undergo conjugation. Cyclins that are made and are involved in G1 were first discovered by their essential role in Start (see ref. 4 for review). Three redundant cyclin genes were originally identified (*CLN1*, *CLN2* and *CLN3*); the messenger RNA and protein levels for *CLN1* and *CLN2* undergo periodic variation with the cell cycle, peaking in late G1, whereas *CLN3*, which is the odd-man-out in several respects, seems to maintain a constant low level of activity throughout the cell cycle (B. Futcher, Cold Spring Harbor Laboratory).

Several groups have simultaneously discovered that the budding yeast G1 cyclins are engaged in a positive feedback loop operating at the level of transcription of the *CLN1* and *CLN2* genes. Fred Cross (Rockefeller Univ.) came across this feedback control in studying a *CLN* mutation that prevents  $\alpha$ -factor arrest of the cell cycle<sup>5</sup>. A dominant mutation of the *CLN3* gene, which encodes a hyperstable protein, both overrides  $\alpha$ -factor arrest at Start and prevents the usually concomitant down-regulation of levels of *CLN1* and *CLN2* mRNA. Further studies revealed that transcription of the *CLN1* and *CLN2* genes depends on the presence of at least one functional *CLN* gene, as well as the *CDC28* gene, and the results suggest that  $\alpha$ -factor indirectly interferes with transcription of *CLN1* and *CLN2* by inhibiting *CLN* function.

Ira Herskowitz (Univ. California San Francisco) and Kim Nasmyth (Inst. Molecular Pathology, Vienna) were led to the same discovery by a different route. Their groups

1. Van de Velde, H., von Hoegen, I., Luo, W., Parnes, J. R. & Thielemans, K. *Nature* **351**, 662–665 (1991).
2. Poo, W.-J., Conrad, L. & Janeway, C. A. Jr *Nature* **332**, 378–380 (1988).
3. Brian, A. A. *Proc. natn. Acad. Sci. U.S.A.* **85**, 564–568 (1988).
4. Hodgkin, P. D., Yamashita, L. C., Coffman, R. L. & Kehry, M. R. *J. Immun.* **145**, 2025–2034 (1990).
5. Noelle, R. J., Daum, J., Bartlett, W. C., McCann, J. & Shepherd, D. M. *J. Immun.* **146**, 1118–1124 (1991).
6. Baluyut, A. R. & Subbarao, B. *J. Molec. cell. Immun.* **4**, 45–57 (1988).
7. Cambier, J. & Lehmann, K. *J. exp. Med.* **170**, 877–886 (1989).
8. Von Hoegen, I., Hsieh, C., Schwarting, R., Francke, U. & Parnes, J. R. *Eur. J. Immun.* (in the press).
9. Subbarao, B. & Mosier, D. E. *J. Immun.* **130**, 2033–2037 (1983).
10. Yakura, H., Shen, F.-W., Kaemmer, M. & Boyse, E. A. *J. exp. Med.* **153**, 129–135 (1981).
11. Springer, T. A. *Nature* **346**, 425–434 (1990).
12. Dang, L. H. & Rock, K. L. *J. Immun.* **146**, 3273–3279 (1991).
13. Linsley, P. S. *et al. J. exp. Med.* **173**, 721–730 (1991).
14. Fraser, J. D., Irving, B. A., Crabtree, G. R. & Weiss, A. *Science* **251**, 313–316 (1991).
15. Lindsten, T., June, C. H., Ledbetter, J. A., Stella, G. & Thompson, C. B. *Science* **244**, 339–343 (1989).
16. Mueller, D. L., Jenkins, M. K. & Schwartz, R. H. *Rev. Immun.* **7**, 445–480 (1989).
17. Koulova, L., Clark, E. A., Shu, G. & Dupont, B. *J. exp. Med.* **173**, 759–762 (1991).
18. Damle, N. K., Linsley, P. S. & Ledbetter, J. A. *Eur. J. Immun.* **21**, 1277–1282 (1991).
19. Imboden, J. B., June, C. H., McCutcheon, M. A. & Ledbetter, J. A. *J. clin. Invest.* **85**, 130–134 (1990).

\* LVI Cold Spring Harbor Symposium, 29 May–5 June 1991.



have both been working on the *SWI4* and *SWI6* genes, which are required for Start (and thus *CDC28*)-dependent transcription of the *HO* gene (which encodes the endonuclease responsible for gene-switching at the mating-type locus — see ref. 6 for review). *SWI4* and *SWI6* turn out to be essential in the budding yeast cell cycle. Nasmyth reported that the functions of the G1 cyclins depend on *SWI4/6*, and that the lethality of *swi4*, *swi6* mutations can be suppressed by constitutive *CLN2* expression<sup>6,7</sup>. Herskowitz described how a screen for suppressors of the lethality of *swi4* mutations at high temperatures selected for the known G1 cyclins *CLN1* and *CLN2*, as well as a putative new G1 cyclin (*CLN4* if verified), *HCS26*<sup>8</sup>. Both Herskowitz and Nasmyth make a case that *SWI4/6* act on the *CLN1* and *CLN2* genes at the level of transcription, and Nasmyth and his colleague Dirick<sup>9</sup> have gone on to confirm that this depends on an active *CDC28* kinase and can be stimulated by *CLN3* activity.

Thus a positive feedback loop exists in which the G1 cyclins activate transcription of their *CLN1* and *CLN2* (and possibly *HCS26/CLN4*) subset by activating, in concert with *CDC28*, *SWI4* and *SWI6* (see Fig. 2). One twist in the tale concerns the apparently distinct properties of *CLN3*, which is not transcriptionally regulated during the cell cycle. Given that  $\alpha$ -factor and *swi4*, *swi6* mutations block *CLN3*:*CDC28* kinase activity, Futcher suggests that *CLN3* may be involved in upstream regulation, acting only by stimulating transcription of downstream *CLN* genes. To account for what would otherwise be the paradoxical viability of *cln1*, *cln2*, *CLN3* cells, Futcher postulates the existence of a fourth G1 cyclin, *CLN4* — which, interestingly, may now have turned up as *HCS26* in Herskowitz's screen.

The positive feedback loop involving the G1 cyclins is reminiscent of the ability of a

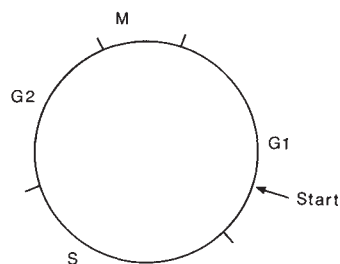


FIG. 1 Phases of the cell cycle in the budding yeast cell cycle.

relatively small amount of MPF post-translationally to activate its own precursor (pre-MPF): in both cases the autoactivation is likely to be important in generating a rapid rise in level of an activity required for a cell-cycle phase transition. As Herskowitz and Gerry Fink (Whitehead Institute, Cambridge, Mass.) both noted, the budding yeast G1 cyclins are targets for inhibition in the intracellular signalling pathway initiated by

$\alpha$ -factor<sup>4</sup>. Herskowitz in particular stressed the formal analogy between components of the pathway and tumour suppressor genes, the absence of which is known to contribute to certain types of cancer, and the ability to dampen down a positive feedback loop may be a general mechanism by which cells are induced to stop cycling.

### Mammalian G1 cyclins

The discoveries of new classes of mammalian cyclins were described at the meeting, some of which are strong candidates for being G1 cyclins like the *CLN* genes of budding yeast. A. Arnold (Massachusetts General Hospital) reported the results recently published in *Nature*<sup>1</sup> and discussed in these pages<sup>2</sup> showing that *PRAD1*, a candidate oncogene, encodes what by sequence is a cyclin with an expression pattern consistent with its being involved in G1. *PRAD1* is overexpressed in a subset of parathyroid tumours as a result of a chromosomal rearrangement fusing the gene to the parathyroid hormone gene. It may be more generally involved in tumorigenesis, as it is located on the long arm of chromosome 11 at band 13, the site of *BCL1* rearrangements in some lymphomas and leukaemias and of gene amplification in a proportion of breast cancers.

Intriguingly, *PRAD1* turns out to be very similar to the mouse cyclin gene *CYL1*, one of a set of putative G1 cyclins that Chuck Sherr (Howard Hughes Medical Institute, Tennessee) and colleagues have found to be induced in macrophages in response to stimulation by the growth factor CSF-1 (ref. 10). CSF-1 is the sole growth factor required for cell-cycle progression by macrophages; in its absence the cells arrest in early G1 and then die. The macrophage receptor for CSF-1 is the product of the proto-oncogene *c-fms*, which in its activated oncogenic form presumably gives a constitutive signal activating expression of the *CYL* genes.

When transiently starved macrophages are restimulated with CSF-1, they synchronously progress from G1 to S phase; they can then proceed to divide in the absence of CSF-1. CSF-1 is required throughout G1 for maintenance of *CYL1* mRNA, and evidence for the functional importance of *CYL* gene expression comes from the correlation between the level of *CYL* protein expression and the proportion of cells entering S phase when this is altered by varying the length of exposure to CSF-1.

The *PRAD1/CYL1* gene has also turned up in screening for new cyclin genes by their ability to complement absence of *CLN* function in budding yeast; in this case the human gene was dubbed cyclin D1 (ref. 11). Steve Reed (Scripps Institute, California) reported that a similar screen identified what, on the basis of their sequences, were identified as three classes of human cyclin genes (cyclins C, D and E). But although the expression patterns are consistent with at least some of these having a role in G1, the complementa-

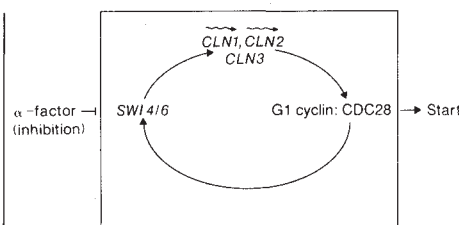


FIG. 2 Positive feedback loop involving budding yeast G1 cyclins. Wavy lines show the genes thought to be activated at the transcriptional level by *SWI4/6*;  $\alpha$  factor is an inhibitor of the feedback loop and behaves as a negative growth factor for yeast. (Adapted from ref. 9).

tion test is clearly not sufficient to define the physiological function of a cyclin.

Little is yet known of the functions of these new classes of mammalian cyclins, although Arnold and colleagues have some evidence that the *PRAD1* gene product can bind to and activate *p34<sup>cdc2</sup>* in clam embryo lysates. It may be, however, that vertebrate G1 cyclins will functionally interact not with *p34<sup>cdc2</sup>* itself but with related kinases now being discovered. Both John Newport (Univ. California San Diego) and Marcel Philippe (Univ. Rennes) reported evidence that the product of a *cdc2*-related gene (*CDC2B* or *Eg-1*) is specifically required for DNA replication in *Xenopus* egg and oocyte extracts. And Ed Harlow (Massachusetts General Hospital Cancer Center, Charlestown) put paid to any remaining hope of simplicity by describing the discovery of ten human genes that, from their sequences, look likely to encode *cdc2*-like protein kinases.

The feeling at the meeting was one of excitement at the stream of new discoveries, tinged with some dismay at the increasing complexity of our picture of how the cell cycle is regulated. Even *cdc2*/MPF may soon fall from its pedestal as an apparently necessary and sufficient inducer of mitosis, with the report by Steve Osmani (Baylor College, Houston) that a second protein kinase, encoded by the *nimA* gene, is required as well as the *cdc2* gene product for initiation of mitosis in *Aspergillus nidulans*. Given the level of research activity, the complexities of the problem should not prove overwhelming. And in unravelling how the periodic events of the cell cycle are coordinated, substantial advances will surely be made in understanding how control of the cell cycle goes awry in cancer. □

Geoffrey North is Deputy Biology Editor of *Nature*.

1. Motokura, T. et al. *Nature* **350**, 512–515 (1991).
2. Hunt, T. *Nature* **350**, 462–463 (1991).
3. Nurse, P. *Nature* **344**, 503–508 (1990).
4. Nasmyth, K. *Cell* **63**, 1117–1120 (1990).
5. Cross, F. & Tinkelenberg, A. H. *Cell* **65**, 875–884 (1991).
6. Herskowitz, I. *Nature* **342**, 749–757 (1989).
7. Nasmyth, K. & Dirick, L. *Cell* (in the press).
8. Ogas, J., Andrews, B. & Herskowitz, I. *Cell* (submitted).
9. Dirick, L. & Nasmyth, K. *Nature* (in the press).
10. Matsushime, H., Roussel, M. F., Ashmun, R. A. & Sherr, C. J. *Cell* **65**, 701–713 (1991).
11. Xiong, Y., Connolly, T., Futcher, B. & Beach, D. *Cell* **65**, 691–699 (1991).

# Our dim and distant past

Richard S. Ellis

The finite velocity of light enables astronomers to become time travellers, probing epochs when the Universe was only a small fraction of its present age. Steidel *et al.*<sup>1</sup> and Thompson and Djorgovski<sup>2</sup> have now discovered what may be the forerunners of normal galaxies at very large distances, seen at a 'look-back time' over half way back to the original Big Bang. By comparing the properties of a representative sample of distant galaxies with those of the present-day population, it ought to be possible to construct a physically consistent picture of galaxy formation and evolution. Because some theories predict a remarkably recent era of galaxy formation, even demonstrating the existence of normal galaxies half way back in time to the Big Bang would represent considerable progress in cosmology.

What is the most effective and reliable way to find distant galaxies? At very large distances, corresponding to redshifts greater than 2 (redshift is the cosmologist's distance indicator representing the Doppler stretching of light caused by the cosmic expansion), or about three-quarters of the way back to the Big Bang, there are plenty of quasars and radio galaxies around. However, these are rare spectacular objects whose physical connection with normal galaxies, such as our own, remains unclear. What is really needed is some information on the equivalent of normal galaxies at large redshifts, and this is why the recent studies may be helpful.

Directly 'slicing' a catalogue of galaxies into different 'time-shells' using increasing redshift windows is not as straightforward as it sounds. Distant objects are only detectable if they are intrinsically luminous, and the extremes of any distribution may not be representative of the underlying population. Such selection biases may distort our view of the distant Universe. For example, the Doppler effect shifts the ultraviolet component of any distant galaxy's spectrum into the optical; this spectral region will only have strong signals if stars are forming in the galaxy. It is easy to see how the Universe might display an apparent increase in the star-formation rate in galaxies viewed at early times — those not sharing such trends simply would not be seen. In any case, systematically searching complete catalogues of faint galaxies has yielded disappointing results; most appear to be relatively local systems. Before a spectrum has been obtained, there is no easy way to know which are the most distant. At current spectroscopic limits, for every galaxy beyond redshift  $z=0.5$ , there are dozens with  $z \approx 0.2-0.3$ ; in short, this is not a very efficient way to find distant sources.

There have been several attempts to circumvent this problem with a bit of lateral thinking. The most established technique is the observation of intervening clouds of

light-absorbing gas revealed in the spectra of high-redshift quasars<sup>3</sup>. The chemical composition and physical nature of some of these clouds suggests that they arise in gaseous envelopes surrounding high-redshift galaxies. The background quasars are used merely as beacons to highlight galaxies which absorb recognizable parts of the spectrum; the peculiar aspects of the unassociated quasar are, of course, irrelevant.

A sizeable sample of absorbing galaxies has been visually identified by direct imaging and spectroscopy in a recent survey by Bergeron and Boissé<sup>4</sup>. The absorber is identified when the galaxy redshift matches that of the absorption lines (in this case, of ionized magnesium) seen in the quasar spectrum. The most distant absorber so identified<sup>5</sup> has a redshift  $z=1.025$  — further than any galaxy found in direct spectral surveys<sup>6-8</sup>. If an absorbing galaxy is a typical common-or-garden example, the technique may offer a promising shortcut for finding samples of galaxies at distant epochs.

Identifying the absorber is an important verification of the method, but obviously becomes difficult beyond  $z \approx 1$  for the same reason that makes the technique useful in cosmology. Bergeron and Boissé's high identification rate is surprising even at  $z \approx 0.5$  because there should be many more intrinsically feeble galaxies per unit volume, each of which should be capable of producing an absorption line in the spectrum of the background quasar without being directly detectable. To explain the success rate, most absorbers must be luminous galaxies embedded in large gaseous envelopes. This suggests that the technique might be usefully pushed to higher redshifts. But the result could also suggest absorbers are not representative of the bulk of the normal population. For example, they might be that subset of galaxies with large envelopes of surrounding gas. If galaxies assemble from merging subunits, as has been proposed to explain the puzzling high numbers of faint blue galaxies (discussed last year in News and Views<sup>9</sup>), it may become increasingly difficult to identify the absorbers at  $z > 1$ . However, a population of dense, neutral-hydrogen absorbers has already been detected<sup>13</sup> out to much higher redshift, suggesting that disks of normal galaxies may well have been in place at early times.

An independent way to leap-frog beyond  $z \approx 1$  is the technique of gravitational lensing. In the 1930s, Fritz Zwicky suggested that massive clusters of galaxies might act as "natural telescopes" producing magnified images of background objects that would otherwise lie undetected. The spectroscopic confirmation that the arc-like structures seen in some rich clusters represent the lensed light of a single background galaxy<sup>10</sup> has led

to much effort in this area. Because an object is lensed only if it happens to lie behind an unrelated cluster, it should also be a serendipitous galaxy. A surprising fraction of arcs are blue and this has been taken as evidence of evolution shared by the overall population. Since the sources producing the arcs must lie behind the lensing cluster, the technique provides a valuable lower limit to the distance of such a blue sample<sup>11</sup>. To determine whether all galaxies behind distant clusters are blue, one should ideally select arcs at infrared wavelengths: here longer-lived stellar populations dominate, thus avoiding the 'ultraviolet' bias discussed earlier. Infrared detector technology has only recently achieved such a capability and it will be interesting to see the results.

There is, however, one worry about the use of lensing in this context. Arcs are detected as extended features above some faint surface brightness threshold. Surface brightness declines very rapidly, as  $(1+z)^4$  in relativistic cosmologies, so that what we can detect in the most distant sources may represent only the very highest-surface-brightness tips of an underlying iceberg. If these tips are generally the star-forming region, we may well overestimate the significance of the blue arcs.

The new results of Steidel *et al.*<sup>1</sup> and Thompson and Djorgovski<sup>2</sup> use one of the potentially safest methods for finding high-redshift galaxies — pure chance. Most spectrographs analyse light along an entrance slit centred around an object of interest. Such long slits sample the surrounding sky regions, enabling the astronomers to make an accurate subtraction of background signals. However, the surface density of faint objects is sufficiently high that there is a significant chance that the slit will lie over one or more serendipitous objects. During long integrations on sources known to be of interest, additional spectra are often gathered.

Thompson and Djorgovski report the discovery of a  $z \approx 1.018$  emission-line galaxy by just such luck. The galaxy bears some resemblance to Bergeron's  $z=1.025$  magnesium absorber. It is another luminous star-forming galaxy that the authors suggest may be representative of the faint blue population dominating the galaxy counts. In a variant of this technique, Steidel *et al.* find a companion galaxy to a quasar at  $z=2.758$ , making it the most distant in this class by a large margin. The object has a spectrum whose redshift is nearly the same as the associated quasar but whose emission-line features indicate that it is more likely to be a galaxy.

To be detected at all, however, any object with  $z > 2$  must be very luminous indeed. It is quite important to understand exactly what is meant by the term 'galaxy'. Quasars are recognized by their very broad emission lines although occasionally dust-shrouded examples with narrower lines have been proposed. The bright  $z=2.286$  infrared source that colleagues and I have discovered (see next week's issue<sup>12</sup>) may fall in this class. The luminosity of Steidel's object is intermediate



between the brightest galaxies witnessed today and a typical quasar. If such sources are representative of a phase most galaxies went through in their past, one might have expected more high-redshift sources to have been found in the complete surveys of faint galaxies. One is tempted to conclude that such high-redshift examples may be rare. Rather like the quasars and radio galaxies themselves, interpreting such objects in the context of present-day galaxies is difficult.

The emission lines seen by Thompson and Djorgovski and by Steidel *et al.* raise a drawback affecting all the methods discussed here to some extent. The redshifts were derived from a single strong emission line, presumed to arise from ionized oxygen (for the  $z=1.018$  galaxy) or neutral hydrogen (for the  $z=2.758$  source). Had the line been absent, either because any emission was Doppler shifted outside the observing spectral window, or because the galaxy simply didn't have the emission lines, the spectrum would have remained featureless (and probably discarded). Clearly there must be a greater chance of noticing a serendipitous emission-line galaxy: other objects would be deemed to have insufficient signal to make any statement.

So, can such distant examples tell us anything reliable about our own history? Not unless we are sure how each selection method works. Unfortunately, each of the new results has some drawbacks. Serendipity is safe if all the data are published, but it is rarely an efficient way to leap-frog an obstacle. Gravitational lensing is an imaginative approach but may reveal unrepresentative portions of galaxies. Most probably, studying the absorbers will remain the most methodical way to break the 'redshift-1 barrier'. Because an absorber is found through the effects of its gaseous mass rather than its emission, selection effects are likely to be far less troublesome. This is probably the most important consideration of all. There is a wealth of absorption line data and pushing the identification programmes to higher redshift seems the most promising way ahead. □

Richard S. Ellis is in the Department of Physics, University of Durham, South Road, Durham DH1 3LE, UK.

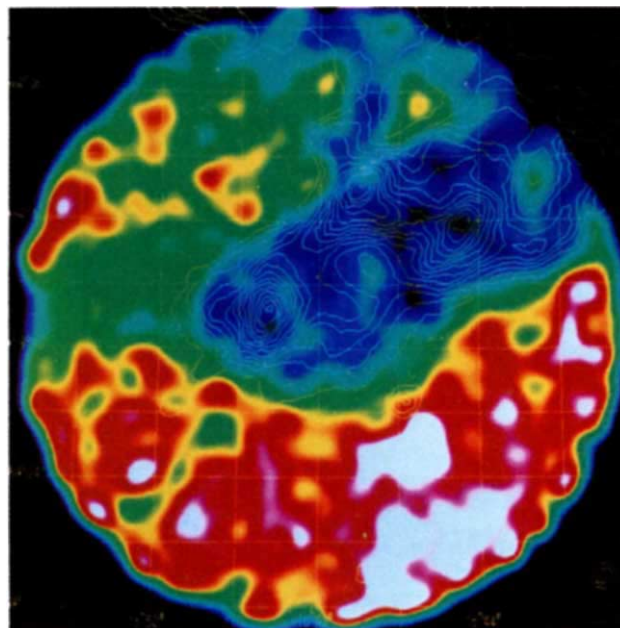
## Draco's silver lining

Steven M. Kahn

OBSERVATIONS with the Roentgen Astronomy Satellite (ROSAT), launched last year, have provided dramatic new evidence for an extensive component of hot gas in the interstellar medium of our Galaxy. Emission from interstellar gas at temperatures of around  $10^6$  K is believed to be responsible for

correlation with the distribution of cooler neutral gas which is suggestive of an absorption effect; that is, X-rays emanating from larger distances are absorbed by foreground neutral clouds.

However, the anticorrelation is much shallower than would be predicted *a priori* on the basis of the expected atomic absorption cross-sections. This discrepancy can be reconciled if the absorbing gas is clumped into clouds that are embedded within the emitting gas<sup>2,4,5</sup>, but the required degree of clumping may be at odds with observational constraints at other wavelengths<sup>6</sup>. Thus, an alternative model<sup>7</sup>, in which all of the hot emitting gas is confined to a Local Bubble, has been more generally favoured. In this picture, the anticorrelation is due not to absorption but to a displacement effect: the neutral material is rarer in directions where the X-ray intensity is high, because it has been displaced by the hot X-ray emitting gas.



D. N. BURROWS

Overcast skies — the soft X-ray background (false colour image) is clearly shadowed by the Draco cloud (indicated by contours of infrared intensity) showing that it originates at galactic distances.

the diffuse background light in the 'soft' X-ray band, 0.1–0.5 keV. ROSAT carries an X-ray telescope with high sensitivity at these energies which was pointed at a prominent cold interstellar cloud, in the constellation Draco, located off the plane of our Galaxy at least 300 parsecs away (the Galaxy is 30,000 parsecs across and 2,000 deep). On page 629 of this issue<sup>1</sup>, Burrows and Mendenhall present ROSAT's soft X-ray image of the background in this region, which reveals a distinct shadow cast by the cloud (see figure). This indicates that a substantial fraction of the observed X-ray emission originates at a distance beyond 300 parsecs, in contradiction to the prevailing model for the soft X-ray background, which holds that all of the emitting hot gas is confined to a 'Local Bubble' within 100 parsecs of the Sun.

The X-ray background at higher energies is known to be extragalactic and is now believed to be produced primarily by a collection of discrete sources at cosmological distances. However, at very low energies, below 0.3 keV, a new component dominates which is thought to be galactic because it correlates very well with galactic latitude. Although this soft component was discovered<sup>2</sup> in the late 1960s, its interpretation is still unclear and has been quite controversial<sup>3</sup>. The intensity exhibits a distinct anti-

This issue has significance in terms of the global structure of the interstellar medium. In particular, recent models have postulated that hot gas produced in explosions of massive stars (supernovae) will eventually interconnect to form a halo extending up to several kiloparsecs above the galactic plane. Some evidence for such a halo has come from ultraviolet observation<sup>8</sup>, but the soft X-ray background could provide strong constraints if the problems with its interpretation can be reconciled.

Observations of isolated, cool interstellar clouds of known distance can provide one of the cleanest methods for determining the origin of the X-ray emitting gas. If a given cloud casts a shadow, then one can measure unambiguously the fraction of X-ray light that emanates from behind it. Experiments along these lines have been attempted in the past, but the results have always been plagued by uncertainties associated with instrumental effects. The ROSAT Observatory provides an excellent new tool for this kind of work: because its instruments have been designed to discriminate true X-ray photons from unrelated particle events, the internal background is low and the sensitivity to variations in the cosmic background is very high.

Burrows and Mendenhall<sup>1</sup> picked a cold

1. Steidel, C. C., Sargent, W. L. W. & Dickinson, M. *Astr. J.* **101**, 1187–1195 (1991).
2. Thompson, D. J. & Djorgovski, S. *Astrophys. J.* **371**, L55–L57 (1991).
3. Bahcall, J. N., Greenstein, J. L. & Sargent, W. L. W. *Astrophys. J.* **153**, 689 (1968).
4. Bergeron, J. & Boissé, P. *Astr. Astrophys.* **243**, 344–366 (1991).
5. Bergeron, J. *ESO Messenger* **62**, 16–17 (1991).
6. Broadhurst, T. J., Ellis, R. S. & Shanks, T. *Mon. Not. R. Astr. Soc.* **235**, 827–857 (1988).
7. Colless, M. M., Ellis, R. S., Taylor, K. & Hook, R. N. *Mon. Not. R. Astr. Soc.* **244**, 408–423 (1990).
8. Lilly, S. J., Cowie, L. I. & Gardner, R. A. *Astrophys. J.* **369**, 79–105 (1991).
9. Ellis, R. S. & Frenk, C. S. *Nature* **346**, 790–791 (1990).
10. Soucail, G., Mellier, Y., Fort, B., Mathez, G. & Cailloux, M. *Astr. Astrophys.* **291**, L19–L21 (1988).
11. Tyson, J. A., Valdes, F. & Wenk, R. A. *Astrophys. J.* **349**, L1–L4 (1990).
12. Rowan-Robinson, M. *et al.* *Nature* (in the press).
13. Wolfe, A. M., Turnshek, D. A., Smith, H. E. & Cohen, R. D. *Astrophys. J. Suppl.* **61**, 249 (1986).

cloud in the constellation Draco which is roughly a degree in size and lies at least 300 parsecs away. It has been mapped in the characteristic radio light of neutral hydrogen as well as in infrared light, which is produced by interstellar dust. In the figure, the X-ray intensity is displayed as a false-colour image overlaid on a contour plot of the infrared map. As can be seen, the X-rays are dim in regions where the infrared is bright, indicating that the cloud is shadowing a significant portion of the X-ray back-ground. Burrows and Mendenhall find that roughly 60–70 per cent of the X-ray light must originate behind the cloud. Snowden *et al.*<sup>9</sup> have also observed the Draco cloud using ROSAT in a scanning mode, and derive similar results.

Although these observations provide important new information, they may raise more questions than they answer. Burrows and Mendenhall argue that the apparent distant contribution to the background cannot be truly pervasive, as would be implied by a halo model, without coming into conflict with earlier observational constraints. For example, if the hot gas extended to similar distances in the direction of the galactic poles (where the amount of neutral gas is low),

then the soft X-ray sky would be much brighter there than is observed. But at the very least, these data do prove that hot gas is present in the Galaxy at much larger distances than previously envisaged. Additional ROSAT shadowing experiments of other clouds will be required to determine whether the direction towards the Draco cloud is anomalous or typical of the X-ray sky. □

Steven M. Kahn is in the Departments of Physics and Astronomy, and the Space Sciences Laboratory, University of California at Berkeley, California 94720, USA.

1. Burrows, D. N. & Mendenhall, J. A. *Nature* **351**, 629–631 (1991).
2. Bowyer, O. S., Field, G. B. & Mack, J. E. *Nature* **217**, 32–34 (1968).
3. McCammon, D. & Sanders, W. T. A. *Rev. Astr. Astrophys.* **28**, 657–688 (1990).
4. Bunner, A. N. *et al. Nature* **223**, 1222–1226 (1969).
5. Jakobsen, P. & Kahn, S. M. *Astrophys. J.* **309**, 682–693 (1986).
6. Jahoda, K., McCammon, D. & Lockman, F. J. *Astrophys. J.* **311**, L57–L61 (1986).
7. Snowden, S. L., Cox, D. P., McCammon, D. & Sanders, W. T. *Astrophys. J.* **354**, 211–219 (1990).
8. Martin, C. & Bowyer, S. *Astrophys. J.* **350**, 242–261 (1990).
9. Snowden, S. L., Mebold, U., Hirth, W., Herbstmeier, U. & Schmitt, J. H. M. *Science* **252**, 1529–1532 (1991).

## ARCHAEOLOGY

# Brilliant — rock art and art rock in Australia

Clive Gamble

Less than 25 years ago, prehistoric stone tools from Australia were described as “crude and rather colourless”<sup>1</sup>. Today, in other hands, they coruscate, casting light not only on their value in survival but also on changes in social values deduced from interpretations of their form and aesthetic appeal.

One such claim is now put forward by Taçon in the latest issue of *Antiquity*<sup>2</sup>. Taçon works in the Kakadu National Park of northern Australia, which is known for its spectacular rock art and which has also been the centre of excavations in the deposits beneath the paintings. Archaeologists have discovered cultural changes in the region over possibly 50,000 years. Moreover, long-term ethnographic enquiries among local Aboriginal communities have added a sacred dimension to the interpretation of stone tools, unearthed in the excavations, that is not possible in other parts of the world. The question for prehistorians is whether this potent Australian combination of rocks, rock art and religious knowledge can be more widely applied to shift the study of ancient stone tools away from just assessing their function to act as a means of investigating changes driven by symbolic behaviour.

Taçon characterizes the different properties of the common rock types, cherts and quartzites in the Kakadu excavations as, respectively, dull and shining. Almost any-

where else this would sound like a story about pretty pebbles on the beach, but not in northern Australia where in an ethnographic study Morphy<sup>3</sup> has identified these properties as the key to understanding the aesthetics of spiritual power. The sources of such power for the Yolngu people of eastern Arnhem Land (which lies to the east of Kakadu) come, ultimately, from the activities of the great ancestral beings of the spiritual Dreamtime. They are contained in objects which have the quality of *bir'yun*, or brilliance. Spear points made from glittering quartzite are a natural example, but brilliance can also be created. Nowhere is it better seen than in the quality captured in Yolngu bark paintings where the fine cross-hatched lines make the images shimmer.

Such power can also be released as tools are made by reducing nodules of stone. White and Jones<sup>4</sup> learned this when studying the Ngilipitji quartzite quarry in eastern Arnhem Land. The stones have power because of their brilliance, which is likened by the Yolngu to the shining fat of an animal. The special killing power of the stone points is accounted for by this perceived property of *djukurr mirri*, having fat.

Morphy argues that *bir'yun* is an effect that operates cross-culturally. It is an essential part of any aesthetic system simply because it produces effects that human beings find stimulating and appealing. The

precise interpretation of its power through associations with objects will of course be culturally specific.

This line of argument may seem to license archaeologists to use alternative classifications of their stone tools — no more lists of scrapers and arrowheads but rather the proportions of rock types arranged on a scale from dull to glittering. Taçon attempts just such an analysis for the past 6,000 years of Kakadu prehistory. He points to the unsuitability of quartzite for making long flakes, and interprets its unexpected dominance as reflecting changes in symbolic behaviour in the region when shimmering rocks outnumber the dull but less intractable red cherts. Taçon also claims that there was an associated change to a more brilliant art style, underlying which were symbolic rather than functional reasons.

Such a magpie model of aesthetics still needs to be adequately tested. Claims for universals in cognitive behaviour to crack prehistoric art systems are a feature of the field<sup>5,6</sup>, but few of them enjoy unqualified support for long. I suspect the concept of brilliance will likewise fade, but not before such work has rightly highlighted the need to reassess the conventional approach to stone tools — in truth, the standard typologies and analyses have revealed little about past behaviour except our own claim to be the great classifiers.

The interest of the magpie model lies not in the glitter factor but in the attempt, either archaeologically or ethnographically, to link the tools with other aspects of the regional landscape and the culture it contains. This point is still most effectively argued by the ‘functional’ camp that Taçon criticizes. In an archaeological study of raw-material rationing in north Queensland, Hiscock<sup>7</sup> shows how technology varies between regions with no change in the implement types. Variation lies in the processes by which flake artefacts are reduced from nodules. This in turn depends upon the suitability of raw material and its availability, so that as transport occurs shapes change in predictable ways — as others have noted, working with European materials<sup>8,9</sup>. By bringing a little colour to the argument, the result is an appreciation of the regional organization of mobile peoples as both sophisticated and subtle. □

Clive Gamble is in the Department of Archaeology, University of Southampton, Southampton SO9 5NH, UK.

1. Clark, J. G. D. *Economic Prehistory* (Cambridge Univ. Press, 1989).
2. Taçon, P. *Antiquity* **65**, 185–198 (1991).
3. Morphy, H. *Man* **24**, 21–40 (1989).
4. Jones, R. & White, N. in *Archaeology with Ethnography* (eds Meehan, B. & Jones, R.) 51–87 (Australian National Univ., Canberra, 1988).
5. Leroi-Gourhan, A. *Préhistoire de l'art Occidental* (Mazenod, Paris, 1965).
6. Lewis-Williams, J. D. & Dowson, T. A. *Curr. Anthropol.* **29**, 201–245 (1988).
7. Hiscock, P. in *Archaeology at Anzias* (ed. Ward, G.) 178–190 (Canberra Arch. Soc., Canberra, 1984).
8. Kuhn, S. L. *J. anthropol. Archaeol.* **10**, 76–106 (1991).
9. Rolland, N. & Dibble, H. *Am. Antiq.* **55**, 480–499 (1990).



# Dads and disomy and disease

Melissa Little, Veronica Van Heyningen and Nicholas Hastie

Two reports, on pages 665 and 667 of this issue<sup>1,2</sup>, show that both a fetal overgrowth syndrome in humans and a related condition in mice are associated with the inheritance of two copies of a homologous genetic region belonging to the father. The wider significance of this link is that it could be yet another case where inheritance depends on the difference in expression between the mother's and the father's genes, that is, on genetic imprinting.

Henry *et al.*<sup>1</sup> demonstrate that in a significant proportion of patients with the fetal overgrowth syndrome known as Beckwith-Wiedemann syndrome (BWS), both copies of a region on the short arm of chromosome 11, 11p15.5, come from the father (paternal disomy/maternal deficiency). And Ferguson-Smith *et al.*<sup>2</sup> show that chimaeric mouse embryos containing cells paternally disomic for the distal part of chromosome 7 are abnormally large, as in BWS. The gene for insulin-like growth factor-2 (*Igf2*) lies in this region and Ferguson-Smith *et al.* show that only the paternal copy is being expressed. Mouse chromosome 7 is homologous to human chromosome 11p15.5. So here we have exclusive paternal expression of an important embryonal growth factor from the very region for which BWS patients are paternally disomic (see figure). Does this make *Igf2* a good candidate for the BWS gene?

One or more genes at 11p15.5 are already known to be involved in several embryonal neoplasms, including Wilms' tumour, hepatoblastoma and rhabdomyosarcoma, and these tumour types occur in 12.5 per cent of BWS patients. If loss of an 11p15.5 allele occurs in these tumours it is always the maternal one. In addition, 11p15 trisomy found in a few BWS patients always results from duplication of the paternal 11p15 allele<sup>1</sup>. Finally, familial BWS is tightly linked to 11p15.5 (ref. 1). Henry *et al.* now provide evidence that some BWS patients carry two copies of the father's 11p15.5 gene. Also, the degree of homozygosity at 11p15.5 in sporadic probands is greater than that in the normal population, suggesting that these patients tend to inherit two copies of the same allele from the father (paternal isodisomy). And this increased homozygosity was greatest for the insulin and *Igf2* loci. The links between 11p15.5 paternal duplication / maternal loss and BWS imply that this region encodes a paternally active growth promoter or a maternally active growth suppressor.

Could *Igf2* be the BWS gene? Because of their chromosomal location it has long been speculated that an increased gene dosage or activity of either insulin or *Igf2* could cause features characteristic of BWS such as neonatal gigantism and hypoglycaemia. Familial BWS is tightly linked to the *Igf2*-insulin

gene cluster<sup>1</sup>. Those infants of diabetic mothers who develop hyperinsulinaemia to cope with their mothers' hyperglycaemia can show the macrosomia, craniofacial and cardiac defects sometimes seen in BWS. But the insulin gene locus is not believed to be imprinted. And increased copies of the insulin or *Igf2* gene have not been detected by dosage studies, although they would not be expected in cases of parental disomy.

De Chiara *et al.*<sup>3</sup> were the first to show that in the mouse only the paternal copy of the *Igf2* gene is actively transcribed. By homologous recombination they created mice with one inactive *Igf2* allele. Offspring of male mice inheriting the *Igf2* allele were 60 per cent normal size and did not express *Igf2*. But offspring of female mice carrying the mutated *Igf2* allele, together with the normal active paternal allele, were no different in size from their littermates and expressed normal levels of *Igf2*. It remains to be seen whether the human *Igf2* gene is imprinted in the same way as the mouse gene.

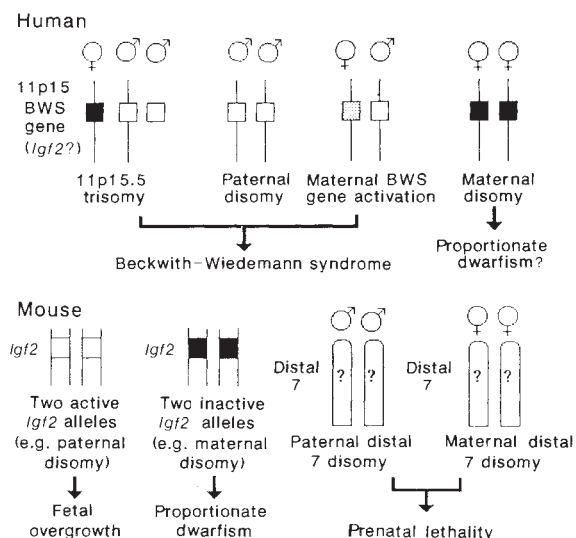
Certainly, an overabundance of *Igf2* messenger RNA is seen in the tumours of BWS patients<sup>4,5</sup>, indicating inappropriate activity of the gene. There has also been one report of a Wilms' tumour patient with an *Igf2* gene containing a structural alteration<sup>6</sup>. If human *Igf2* is similarly imprinted, duplication of the active paternal allele, whether by 11p15 trisomy or paternal disomy, could explain the features of BWS. The maternally imprinted *Igf2* receptor may also be involved in controlling *Igf2* levels<sup>7</sup>. So it will be interesting to see whether some patients show maternal allele loss or paternal disomy for the maternally active *Igf2* receptor gene, which lies at 6q21-27.

Mouse embryos with constitutional distal chromosome 7 disomy, maternal or paternal, die before birth<sup>2</sup>; yet mice homozygous for an inactive *Igf2* gene are viable, although small<sup>3</sup>. So perhaps the distal region of mouse chromosome 7 contains imprinted genes other than *Igf2* that are involved in this lethality. These could be homologous to other human 11p15.5 genes. Perhaps the 11p15.5 region is full of paternally active growth genes, all equally likely to be responsible for BWS. This does not seem to be the case. For example, the mouse *H19* gene, which lies close to *Igf2* in both mouse and man, is expressed from the

maternal allele only<sup>8</sup>. Added to that, the insulin gene, which is situated next to the *Igf2* gene, is not imprinted<sup>3</sup>. Thus imprinting of somatic chromosomes does not span large blocks of genes. Also, adjacent genes are not necessarily all imprinted and those that are may show opposite parental imprinting patterns. That *Igf2* is the BWS gene is therefore more likely, as it is paternally active, growth-promoting and in the right place.

Two human diseases, Prader-Willi and Angelman syndromes, are possibly examples of reciprocal deletion syndromes involving imprinted loci. Prader-Willi syndrome, involving hypotonia and hyperphagia, is associated with paternal allele deletion at 15q11-13 (ref. 9). In contrast, Angelman syndrome patients (puppet children), who show repetitive ataxic movement and seizures, have maternal deletions of the same region<sup>10</sup>. In Prader-Willi syndrome, 15q11-13 deletion is not always seen and the disease can result from maternal isodisomy (two copies of the same allele from the mother) or heterodisomy (one copy of each maternal allele), again resulting in the loss of the paternal copy<sup>11</sup>.

The reciprocal genetics may occur in Angelman syndrome, but has not yet been reported. De Chiara *et al.*<sup>3</sup> found that the normally proportioned dwarfism seen in mice lacking *Igf2* activity persisted throughout their adult life. The findings so far indicate that the BWS gene encodes a growth promoter, possibly *Igf2* itself. Thus,



A comparison between duplications of homologous regions in mouse and humans. Beckwith-Wiedemann syndrome (BWS) has now been associated with duplication of paternal 11p15.5 by trisomy, involving two copies of the paternal alleles, and by paternal disomy. This may be due to exclusive paternal activity of a growth-promoting gene. Thus, the disease could also arise if the maternal allele is inappropriately activated. Part of the distal region of chromosome 7 in the mouse is homologous to human 11p15.5, including the insulin-like growth factor-2 gene (*Igf2*). Only the paternal allele of *Igf2* is actively transcribed in the mouse. Two active copies of *Igf2* result in a fetal overgrowth phenotype similar to that seen in BWS. Paternal and maternal disomy for the distal region of chromosome 7 is lethal owing to the effect of other imprinted genes in this region.

in humans, the genetics underlying proportional dwarfism may be the reciprocal of those of BWS. It will be interesting to look for 11p15.5 maternal disomy in individuals affected by the first of these.

Could the BWS gene be a growth suppressor rather than a growth promoter gene? Loss of a suppressor gene may explain the frequent occurrence of tumours with BWS. Microcell transfer studies with radiation-damaged chromosomes suggest that a tumour suppressor gene exists at 11p15 (ref. 12). If, for example, this growth suppressor gene were maternally active, such as *H19*, then paternal duplication or maternal deficiency would result in its loss of function, and could lead to BWS. But in 11p15 trisomic BWS patients there is duplication of the paternal allele as well as retention of the maternal chromosome.

It would seem, therefore, that the paternal duplication, not loss of the maternal allele, leads to BWS. There may in fact be two closely linked genes, one encoding a growth promoter and one a growth suppressor, imprinted in opposing directions, so that only BWS individuals maternally deficient for the tumour suppressor gene also develop a tumour. If so, the 11p15.5 trisomic patients should still have the active allele of the suppressor gene and would therefore be expected to be less likely to develop tumours. Indeed, of the few known trisomic BWS patients, most have features such as hypoglycaemia, craniofacial abnormalities and cardiac defects, but few develop tumours<sup>13</sup>. Trisomic BWS patients will also have three copies of nearby, unimprinted loci, such as that encoding insulin, which may explain why some show the same cardiac and craniofacial defects as hyperinsulinaemic babies.

We have suggested that nonrandom maternal allele loss in tumours of BWS patients may result from the coincident loss of a maternally active tumour suppressor gene, or that the BWS gene and the 11p15 embryonal tumour gene(s) are one and the same, and overexpression of a growth promoter gene or oncogene also initiates tumour formation. If the latter is the case, then in 11p15-related embryonal tumours, particularly when associated with BWS, allele loss should result from either somatic recombination or chromosome loss and reduplication, but not from hemizygosity. Although hemizygosity is less frequent, cases have been found in the tumours of BWS patients<sup>14</sup>. Therefore, coincident loss of a maternally active tumour suppressor gene is more likely to be a factor in BWS. We have already mentioned the maternally active *H19* gene. This gene is closely linked to *Igf2* and causes late prenatal lethality when maternally disomic. Could this be the 11p15.5 tumour suppressor gene?

For Wilms' tumour, the most common neoplasm in BWS patients, the loss of both copies of an 11p13 tumour suppressor gene may not be the only pathway to tumorigenesis. At least two other loci, including

11p15, could be involved, as a subset of Wilms' tumour patients show maternal allele loss specific to 11p15 and no linkage has been found between familial Wilms' tumour and 11p13 or 11p15. It is possible that a deletion or mutation at 11p13 followed by a second at 11p15, in the BWS gene or an adjacent tumour suppressor gene, may initiate tumour formation in some Wilms' tumour patients. Conversely, the frequency of Wilms' tumour may be high in BWS patients because the constitutional mutation (deletion) leading to BWS increases the number of cells in which a second mutation at either 11p15 or 11p13 will lead to a tumour.

Not all the individuals investigated by Henry *et al.* show paternal disomy. But the disomy could exist over a short 11p15.5 region. Alternatively, some BWS patients could be mosaics, with paternal disomy occurring only in some tissues. This may explain the many incomplete forms of BWS. Wilms' tumour itself may sometimes represent an incomplete form of BWS, mosaicism for the BWS mutation being found only in the kidney.

The final answer to the question of BWS, with and without neoplasia, is yet to come. But for the time being it seems that, at this locus, two copies from dad means big. □

Melissa Little, Veronica Van Heyningen and Nicholas Hastie are in the MRC Human Genetics Unit, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, UK.

1. Henry, I. *et al.* *Nature* **351**, 665-667 (1991).
2. Ferguson-Smith, A.C., Cattanach, B. M., Barton, S. C., Beechey, C. V. & Surani, M.A. *Nature* **351**, 667-670 (1991).
3. De Chiara, T. M., Robertson, E. J. & Efstratiadis, A. *Cell* **64**, 849-859 (1991).
4. Reeve, A. E., Eccles, M. R., Wilkins, R. J., Bell, G. I. & Millow, L. J. *Nature* **317**, 258-260 (1985).
5. Scott, J. *et al.* *Nature* **317**, 260-262 (1985).
6. Irminger, J.-C., Schoenle, E. J., Briner, J., Humbel, R. E. *Eur. J. Pediatr.* **148**, 620-623 (1989).
7. Bartolomei, M. S., Zemel, S. & Tilghman, S. M. *Nature*, **351**, 153-155 (1991).
8. Barlow, D. P., Stoger, R., Herrmann, B. G., Saito, K. & Schweifer, N. *Nature* **349**, 84-87 (1990).
9. Butler, M. G., Meany, F. J. & Palmer, C. G. *Am. J. med. Genet.* **23**, 793-809 (1986).
10. Knoll, J. H. M., Nicholls, R. D., Magenis, R. E., Graham, J. M., Lalande, M. & Latt, S. A. *Am. J. med. Genet.* **32**, 285-290 (1989).
11. Nicholls, R. D., Knoll, J. H. M., Butler, M. G., Karem, S. & Lalande, M. *Nature* **342**, 281-285 (1989).
12. Stanbridge, E. J. & Dowdy, S. F. *Hereditary Tumors* Vol. 83 141-151 (Serono Symposia Publications, 1991).
13. Turleau, C. & de Grouchy, J. *Ann. Genet.* **28**, 93-96 (1985).
14. Mannens, M. *et al.* *Hum. Genet.* **81**, 41-48 (1988).

## Correction

In John Gallop's New and Views article (*Nature* **350**, 465; 1991) on increased critical current densities in films of high-temperature superconductor, the first author of the study described should have been named as H. Jiang not W. Jiang. In the description of the method for fabricating sub-microscopic superconducting bridges, the text should have stated that films 500 nm thick were chemically etched to produce patterns with a tolerance of 20-50 µm, subsequently milled with ion beams.

# Nuclear ballistics

HIGH-ENERGY physics is misleadingly named. A million particles at 1 TeV have between them less than a joule of energy. The real problem is concentrating modest amounts of energy into such a small number of particles. Modern particle accelerators manage this in a stupefyingly complex and expensive manner. Daedalus now plans to do it directly, with high explosive.

How to transfer all the energy of a big explosion into a tiny beam of particles? The problem is similar to that of transferring all the energy of a large mass of rocket fuel into a tiny payload. A practical rocket does this in a few discrete stages. Daedalus once proposed the optimal limiting case, an infinitely staged rocket. It simply burned continuously away into an ever-smaller copy of itself. In theory, its ultimate infinitesimal tip could reach infinite velocity.

So intrepid DREADCO chemists are scaling this idea down to particle-physics energies. They are cutting and polishing single crystals of high explosive into a carefully calculated shape: a perfect cone with the sharpest possible point. The cone will then be detonated uniformly all over its flat base, by a short, intense laser pulse.

As the detonation wave travels up the cone, the reaction products blasting away from its base will accelerate it point-first, rocket-fashion. As the explosion progresses and the cone shrinks and accelerates, more and more of the explosion energy will be transferred to a smaller and smaller section of cone. A single-crystal explosive, detonating by coherent molecular transmission rather than thermally, should be able to explode to the last molecule. As the explosion propagates through the micron-sized tip of the cone, almost all the energy imparted by the entire explosion will be concentrated in the reaction products of the final few billion molecules. They will then be travelling at many TeV.

The simplest particle experiment would merely fire such a beam into a stationary target. More elegantly, two such cones could be aligned facing each other, and fired simultaneously to make their beams collide. A sort of machine-gun device, feeding a supply of cones into a massive and accurately aligned jig, and firing them in rapid succession, could automate the experiment. Experimental particle-physics would then make as much noise as a small war (at present, it merely costs as much).

There may be another military connection, however. Imagine a close-packed sphere of explosive cones, all pointing inwards to a tiny fusion target at the centre. Firing the cones might concentrate enough energy on the target to give a pure fusion explosion with no fallout. This elegant weapon resembles the original fission bomb, but is environmentally far more acceptable.

David Jones



# Volcano warning needed

SIR — The recent eruptive activity at Unzen volcano and its tragic consequences emphasize how little the hazards associated with silicic lava flows and domes are appreciated, both by civil defence agencies and volcanologists. Local experts awaited seismic warnings that gas-rich magma was rising through the main conduit; when these warnings failed to materialize, reporters, geologists and citizens of nearby towns assumed that imminent danger had subsided. Although we do not yet know the exact circumstances leading to the deaths of several dozen people, most likely the front of the young lava flow collapsed, allowing gases from the hot interior to expand into an explosive pyroclastic flow. Last month at Colima volcano in Mexico, collapse of a similar flow some distance from the vent also sent block and ash flows many kilometres down a steep valley; fortunately no geologists or civilians were in its path. The same type of activity at Santa Maria volcano in Guatemala over the past year and a half has led to evacuation of many villages in the surrounding valleys, and was probably responsible for killing five thousand people in 1929. Referred to as Merapi-type behaviour, this kind of flow-front collapse has been documented around the world throughout this century.

The failure to recognize the dangers associated with slowly moving viscous lava flows comes partly from the company these extrusions keep. The emergence of silicic lavas is frequently preceded or accompanied by explosive eruptions of magma directly from a vent, as at Mount St Helens in 1980. The simple view of these volcanic systems is that the source magma body is crudely stratified with volatiles; the most water-rich magma at the top erupts first explosively, followed by quieter effusions of drier lava. By the time a viscous dome begins to form, the probability of explosive products coming directly from the vent is markedly reduced. However, as documented by Rose *et al.*<sup>1</sup> and explored further by Fink and Manley<sup>2</sup>, the explosive collapse of a silicic flow up to several kilometres from a vent may generate block and ash flows capable of moving rapidly down valleys, scorching everything in their paths.

The reason these late-stage explosions take place relates to the ability of a flow to retain the relatively small amounts of volatiles it contains when it emerges from a vent. Viscous flows with thick carapaces may hold and even concentrate these gases (mostly water, but also sulphur, chlorine and fluorine). Crystallization of the flow interior may expel additional gases. If concentrations become high enough in flows on shallow slopes, volatile pressure may drive explosions which form craters in the flow's upper

surface. For flows moving down steep slopes, as at Unzen, Colima and Santa Maria, slumping of the front is common. Rapid exposure and decompression of the hot, gas-rich interior can then generate pyroclastic flows with little or no advance warning.

The message for volcanologists, reporters and civil defence officials is that active lava flows of intermediate to felsic compositions should always be viewed as threats to all areas immediately downslope, not just when

seismicity suggests the arrival of new magma to the surface, or when rainfall makes mud-flows likely. The actual timing of collapse cannot be predicted, although it may be facilitated by earthquakes. Field, laboratory and drilling studies of the volatile contents and emplacement process of older silicic domes<sup>3</sup> may reveal patterns and clues that can be used to assist future predictions.

JONATHAN FINK

Department of Geology,  
Arizona State University,  
Tempe,  
Arizona 85287-1404, USA

## Is the viceroy a batesian mimic?

SIR — Mimicry theory proposes that prey species evolve colour patterns similar to those of unpalatable models because they gain protection through the experienced predator's propensity to generalize learnt aversive associations to prey with similar appearances. In a müllerian mimicry ring, participant species are all unpalatable co-mimics, but in batesian mimicry perfectly palatable prey have evolved mimetic coloration, gaining protection by confusing predators that have had aversive experiences with unpalatable models. The viceroy butterfly, *Limenitis archippus* (Cramer), has long been regarded as a classic example of batesian mimicry, being apparently palatable yet looking similar to two unpalatable danaine models: the monarch, *Danaus plexippus* (L.), and queen, *Danaus gilippus* (Cramer). Ritland and Brower<sup>1</sup> claim to have demonstrated that viceroys are not batesian mimics at all, but unpalatable müllerian co-mimics of the danaines. But there is another interpretation of the results they present that is consistent with the view that viceroys are palatable batesian mimics.

Ritland and Brower presented individual wild-caught red-winged blackbirds, *Agelaius phoeniceus* (L.) with abdomens of monarchs (model), queens (model), viceroys (putative mimic) and known palatable controls. Birds showed as much aversion to viceroys as to monarchs, and significantly more than to queens, a result taken to indicate that viceroys are unpalatable. But because all the birds received all types of butterfly, they may not have reacted to each in a way that was independent of experience with other species encountered during the experiment (or, in fact, before capture). Presenting only abdomens ensured that birds could not generalize any learnt aversion across the known similarities between species in wing pattern, but visual characteristics are not the only ones over which generalization is possible. The monarch's cardenolide toxins also have a bitter taste<sup>2</sup> which may become a conditioned signal of toxicity in its own right<sup>3</sup>. If the viceroy also tastes bitter, or similar to the monarch in other ways, then even if it is palatable, birds might still find it aversive because its taste reminds them of the monarch. We should not confuse 'taste' with

'unpalatability'<sup>2</sup>. Because 35 per cent of viceroys were apparently 'taste-rejected', evidence on whether they taste similar to monarchs or queens would be illuminating.

Monarchs contain distinctive-smelling pyrazines<sup>4</sup>, which, though not directly aversive to birds, can be detected by them, and can become conditioned signals of aversive substances<sup>5</sup>. Viceroys are said to smell like monarchs to humans<sup>4</sup>. If this is also true for birds, then birds that have experienced monarchs during or before the experiment might treat viceroys as if they were unpalatable even if they are not.

Wing removal does not control for the potentially confounding effects of similarities in olfactory or taste cues: individual naïve birds should be given trials of just one butterfly type, or force-fed with encapsulated butterfly material as has been done for monarchs and queens<sup>5</sup>.

If this alternative interpretation is correct, and the viceroy is a batesian mimic after all, an interesting conclusion follows. Viceroys would have to be mimicking monarchs, and perhaps queens, not just visually, but in at least one other sensory modality (taste or smell) as well. Where multiple mimicry is aimed at a single type of predator (such as birds) it may present novel problems for the evolutionary dynamics of mimicry.

TIM GUILFORD

Department of Zoology,  
University of Oxford,  
Oxford OX1 3PS, UK

1. Ritland, D. B. & Brower, L. P. *Nature* **350**, 497–498 (1991).
2. Brower, L. P. in *The Biology of Butterflies* (eds Vane-Wright, R. I. & Ackery, P. R.) 109–134 (Academic, London, 1984).
3. Brower, L. P. *Scient. Am.* **220** (2), 22–29 (1969).
4. Rothschild, M., Moore, B. P. & Brown, V. *Biol. J. Linn. Soc.* **23**, 375–380 (1984).
5. Guilford, T. C., Nicol, C. J., Rothschild, M. & Moore, B. P. *Biol. J. Linn. Soc.* **31**, 113–128 (1987).

SIR — I read with great pleasure that Jane Brower's seminal paper<sup>1</sup> published in 1958 in which she concluded that the viceroy butterfly was not "either a batesian or müllerian mimic in the classical sense" has at last been confirmed<sup>2</sup>. Most of us working in this delectably controversial field have agreed with Brower, but despite the lapse of 30 years we have all, so far, failed to isolate, let alone identify, the deterrent principle

1. Fink, J. H. & Manley, C. R. *IAVCEI Proc. Volcanol.* **1**, 169–179 (1989).  
2. Rose, W. I., Pearson, T. & Bonis, S. *Bull. Volcanol.* **40**, 53–70 (1977).  
3. Anderson, S. W. & Fink, J. H. *Nature* **341**, 521–523 (1989).

— not for want of trying.

We failed to find salicin (present in the food plant) in the tissues of the viceroy (M. R. and R. Nash, unpublished); nor was pyrazine present in its offensive, aposematic odour<sup>3</sup>. However, we did find it contained a high concentration of carotenoids (938.7 µg per g) characteristic of models rather than mimics<sup>4</sup> and we remarked once again that "the Viceroy may well be a müllerian rather than a batesian mimic".

The identification of self-secreted active deterrents in Lepidoptera, some of which also sequester toxins from their food plants, is rather difficult. We found a lethal protein in *Arctia caja* (which sequesters and stores cardiac glycosides and pyrrolizidin alkaloids from its food plants) which we named cajan<sup>5</sup> and another lethal substance in white butterflies (which sequester mustard-oil glycosides from Cruciferae) which we named pierin<sup>6</sup>. But who is going to hit the jackpot by isolating and identifying limenitit?<sup>7</sup>

MIRIAM ROTHSCHILD

Ashton Wold,  
Peterborough PE8 5LZ, UK

1. Brower, J. V. L. *Evolution* **12**, 32–47 (1958).
2. Ritland, D. B. & Brower, L. P. *Nature* **350**, 497 (1991).
3. Moore, B. P., Vance Brown, W. & Rothschild, M. *Chemoecology* **1**, 43–51 (1990).
4. Rothschild, M., Mummery, R. & Farrell, C. *Biol. J. Linn. Soc.* **28**, 359–372 (1986).
5. Hsiao, C., Hsiao, T. & Rothschild, M. *Toxicon* **18**, 291–299 (1980).
6. Marsh, N. & Rothschild, M. *J. Zool., Lond.* **174**, 89–122 (1974).

SIR — Vane-Wright suggests<sup>1</sup> that one of the most telling points against batesian mimicry is likelihood, on the grounds that chemical defence is more likely to evolve because it provides more reliable protection. We are not aware of any evidence that this argument is valid. Evolution is opportunistic and has to make do with the genetic variation that is actually available, and biochemical and ecological constraints on the synthesis and storage of chemical defences may, for all we know, greatly exceed those affecting colour patterns. No doubt comparative and phylogenetic studies could establish that chemical defences do evolve more frequently than mimetic resemblances. But this is a historical and statistical necessity as unpalatability must exist before mimicry can evolve, and the evolution of mimicry is itself an improbable event.

Ritland and Brower note the obvious dangers of extrapolating from a laboratory experiment to the field<sup>2</sup>. It would clearly be highly desirable to use field tests to discriminate between batesian and müllerian mimicry. Such tests are possible if frequency-dependent predation of different colour forms can be detected. In müllerian mimicry, predation is negatively frequency-dependent (rare forms attacked) whereas in batesian mimicry it is positively frequency-dependent (common forms attacked). We believe that the use of this criterion has been wrongly neglected in favour of laboratory experiments and chemical investigations. Batesian<sup>3</sup>

and müllerian<sup>4</sup> mimicry can be distinguished in this way. Where naturally occurring colour variants are lacking, artificially altered butterflies can be used<sup>4</sup>.

Finally, we wish to draw attention to the existence of rule-breaking mimics<sup>5</sup>, in which there is widespread biogeographical mismatching in the distributions of mimics and models. The study of mimicry has long since reached the point where the exception is far more interesting than the rule, and we believe that further investigation of such cases may reveal much about the circumstances under which functional mimicry can evolve.

CYRIL A. CLARKE

Department of Genetics &  
Microbiology,  
University of Liverpool,  
Liverpool L69 3BX, UK

F. M. M. CLARKE

43 Caldys Road,  
West Kirby, Wirral L48 2HF, UK

IAN J. GORDON

Department of Zoology,  
University of Nairobi,  
Box 30197, Nairobi, Kenya

NEVILLE A. MARSH

Division of Biomedical Sciences,  
King's College London,  
Strand, London WC2R 2LS, UK

1. Vane-Wright, R. I. *Nature* **350**, 460–461 (1991).
2. Ritland, D. B. & Brower, L. P. *Nature* **350**, 497–498 (1991).
3. Gordon, I. J. *Biol. J. Linn. Soc.* **31**, 1–23 (1987).
4. Benson, W. W. *Science* **176**, 936–939 (1972).
5. Clarke, C. A., Clarke, F. M. M., Gordon, I. J. & Marsh, N. A. *Biol. J. Linn. Soc.* **37**, 359–365 (1989).

## Virus ecology

SIR — Suttle *et al.*<sup>1</sup> suggest that in addition to grazing and limitation of nutrients, infection of phytoplankton by marine viruses could be a factor regulating their production. The evidence for this hypothesis is the huge amounts of virus particles found in marine waters, and the finding that up to 20-fold concentrations of such can reduce the photosynthesis of marine phytoplankton under experimental conditions<sup>1</sup>. Although this hypothesis is attractive, we think that one important aspect of this phenomenon has not been considered. When a new, highly virulent virus type is introduced into a population of initially susceptible individuals, an enormous selection pressure for virus-resistant variants is generated. A well-known example of this phenomenon is the artificial introduction of myxoma virus into Australia to help control the rabbit pest problem<sup>2</sup>. In this giant field experiment, genetic resistance to myxomatosis increased so that the mortality rate after infection with a particular strain of virus decreased from 90 to 25 per cent within a few years.

This phenomenon is not restricted to virus–animal systems. Shortly after his discovery of bacteriophages<sup>3</sup>, d'Herelle suggested that bacterial viruses might serve a useful therapeutic purpose; but he was never able to prove his idea experimentally<sup>1</sup>. The

suggestion that exogenous marine viruses should continuously be able to kill a large proportion of a phytoplankton community seems to us as unlikely as the idea that bacteriophages should eliminate pathogenic or other bacteria within a given ecological niche. Therefore, if marine viruses are involved in the control of primary plankton production, this probably occurs only if a new virulent virus strain emerges, and the effects on primary production would most likely only be transient, owing to the rapid evolution and reproduction of resistant phytoplankton strains.

SIGVARD OLOFSSON

Department of Virology,  
University of Göteborg,  
Guldhedsgatan 10B,  
S-413 46 Göteborg, Sweden

STAFFAN KJELLEBERG

Department of General and Marine  
Microbiology,  
University of Göteborg,  
Carl Skottsbergs Gata 22,  
S-413 19 Göteborg,  
Sweden

SUTTLE *ET AL.* REPLY — Olofsson and Kjelleberg doubt that viruses could reduce primary productivity because of selection for virus-resistant variants. This point is an important aspect of viral ecology and we agree that resistant phytoplankton would arise if put under strong selective pressure by a viral pathogen.

The greatest impact of viruses on photosynthetic rates probably occurs in specific situations, such as during the demise of large monospecific phytoplankton blooms. Resistant variants would not prevent a transitory decrease in productivity as the infection propagated, but would presumably protect the host species from local extinction. In non-bloom situations, viruses probably remove a portion of the primary productivity, as lytic viruses occur in sea water and must be produced at the expense of hosts. As viral infections propagate rapidly when host density becomes high<sup>5</sup>, viruses may also indirectly affect primary productivity by modifying community structure. In other situations viruses could stimulate primary productivity by causing cell lysis and enhancing the rates of nutrient recycling, analogous to the effect of grazers in some planktonic communities<sup>6</sup>. Observations that aquatic viruses infect particular hosts over wide geographic areas<sup>7,8</sup>, that a significant portion of marine cyano-

1. Suttle, C. A., Chan, A. M. & Cottrell, M. T. *Nature* **347**, 467–469 (1990).
2. Fenner, F. & White, D. O. *Medical Virology* 142–158, (Academic, New York, 1970).
3. d'Herelle, F. *Le Bactériophage* (Masson, Paris, 1921).
4. Fields, B. N. & Knipe, D. N. *Fields Virology* (eds Fields B. N. & Knipe D.), 3–7, (Raven, New York, 1990).
5. Wiggins, B. A. & Alexander, M. *Appl. Environ. Microbiol.* **49**, 19–23 (1985).
6. Lehman, J. T. *Limnol. Oceanogr.* **25**, 620–632 (1980).
7. Moebius, K. & Nattkemper, H. *Helgolander Meeresunters.* **36**, 357–373 (1981).
8. Suttle, C. A., Chan, A. M. & Cottrell, M. T. *Appl. Environ. Microbiol.* **57**, 721–726 (1991).
9. Proctor, L. M. & Fuhrman, J. A. *Nature* **343**, 60–62 (1990).



bacteria are infected with viruses<sup>9</sup>, and that titres of viral pathogens to specific hosts are highly variable (our unpublished results) emphasize the dynamic interactions of host-virus systems in the sea. But the role of host resistance is likely to be limited, because marine virus and phytoplankton communities are generally diverse with only a small proportion of the total numbers made up of any given pathogen or host. So the low probability of a virus encountering a suitable host minimizes selection for host resistance relative to other factors.

We would also like to point out that we did not conclude that viruses caused the observed decreases in photosynthetic rates.

CURTIS A. SUTTLE

AMY M. CHAN

MATTHEW T. COTTRELL

Marine Science Institute,  
University of Texas at Austin,  
Port Aransas,  
Texas 78373-1267, USA

## Original mud

SIR — The recent illustrations<sup>1</sup> of our proposed chordate ancestors demonstrate remarkable morphological similarities to the class Rotifera, whose contemporary members inhabit both fresh and saline waters. The figure shows the close resemblance between the fossil carpodid, mitrate and hemichordate on the left-hand side, and several species of rotifera on the right. There are particular

parallels between the rotifer tail, which has several flexible segments and a neural ganglion at its base, and that of the fossils. Rotifera use these caudal appendages to creep over or fasten onto surfaces when they are not free-swimming.

There are considerable differences in size between these creatures, however: the fossilized species are of the order of 1–3 cm long, whereas rotifera seldom exceed 0.5 mm in length. Their respective habitats differ too: the carpodids and mitrates inhabited marine mud, and probably seldom moved freely in open water, but the rotifera illustrated here live predominantly on top of such muddy surfaces. One might explain this coincidence of structure as a case of convergent evolution, as the fossil species had more sophisticated organs and cuticular development. Could this example of convergent evolution therefore be attributed to the rotifer's adaptation to water's greater relative viscosity (as defined by the Reynolds's number<sup>2</sup>), leading to the development of similar structures as observed in larger creatures living in thick mud?

C. A. MICHIE

Imperial Cancer Research Fund,  
University College and  
Middlesex Schools of Medicine,  
91 Riding House Street,  
London W1P 8BT, UK

1. Paul, C. *Nature* **348**, 680–681 (1990).

2. Reynolds, O. *Phil. Trans. R. Soc.* **174**, 174–188 (1883).

## Formation of membranes

SIR — The interest of theoretical physicists in biomembranes has yielded new understanding in this important field, where on the one side so much is known and on the other so little is understood<sup>1,2</sup>. But despite their successes in explaining various phenomena, thermodynamic approaches provide mainly an overall, static picture of the systems.

For understanding the mechanisms, transformations and other dynamic phenomena on the molecular scale these approaches should be combined, I think, with the kinetic ones. These, I believe, originate predominantly in the 'structural paradox' of the aggregates: because of the aggregates' topology, only some of the molecules are exposed to the changes in the exterior; while these (surface) molecules are trying to minimize their free energy they prevent the molecules in the interior of the structure in doing the same. Because permeability rates can be very low this imposes various gradients (hydration, temperature, pressure, concentration of some solutes which can all influence the area of polar head groups, relative mobility of hydrocarbon chains and therefore expansivities of monolayers in a bilayer<sup>3</sup>, or in stacks of smectic bilayers), which, in turn, dictate the spatial and temporal evolution of the system.

Larger surface areas and/or expansivities of the outer monolayers (or layers in an aggregate) induce nonzero curvature which causes not only the shape fluctuations of cells or vesicles<sup>3</sup>, but is also the driving force for the formation of vesicles. Simply, in various vesicle/liposome preparation methods, the curvature is induced either in the polar or nonpolar part of the bilayer, or on the rim of bilayered fragments by various treatments of amphiphile film, dispersion, emulsion or solution. It seems that these gradients allow the systems to be brought over energy barriers into metastable states, as vesicles are generally thermodynamically unstable due to their curvature<sup>1,2,4</sup>. In other words, due to these gradients some of the energy, which can be released into the system by an exothermic process and which would normally dissipate as heat, can be stored as conformational free energy in the particle.

With passing time these gradients dissipate and a homogeneous liposome colloidal solution transforms into a heterogeneous dispersion of fully hydrated aggregates with large curvature radii in excess water<sup>4</sup>. These transformations are again a consequence of the interplay of thermodynamic and kinetic factors — I believe that for the thorough understanding of the behaviour of amphiphiles in aqueous solutions as well as vesicle formation in living cells, both approaches should be taken into account.

D. D. LASIC

Department of Research,  
Liposome Technology, Inc.,  
1050 Hamilton Court,  
Menlo Park, California 94025, USA

1. Lipowsky, R. *Nature* **349**, 475–481 (1991).

2. Lasic, D. D. *Biochem. J.* **256**, 1–11 (1988).

3. Berndt, K., Kas, J., Lipowsky, R., Sackmann, E. & Seifert, U. *Europhys. Lett.* **13**, 659–664 (1990).

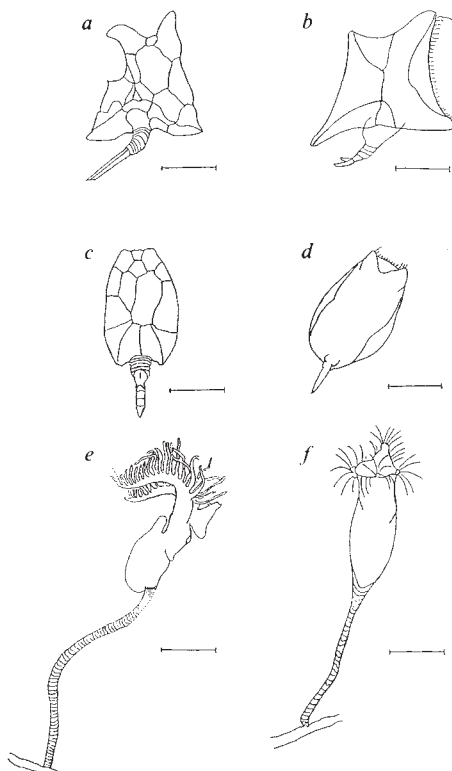
4. Lasic, D. D. *J. Colloid Interface Sci.* **140**, 302–304 (1990).

## Cannibalism in the Neolithic

SIR — Bahn in his News and Views article<sup>1</sup> suggests that cannibalism did not occur at the Neolithic site of Fontbrégoua in southern France, arguing that secondary burial practices involving defleshing of partly decomposed bodies can produce the same results as alleged cannibalism. But the burial hypothesis does not adequately account for all the evidence from this site.

Throughout the fifth and fourth millennia BC Fontbrégoua was used as a temporary habitation site by small groups of hunters who kept herds of sheep in pens inside the cave. Most of the butchering was done in the cave and bones were discarded in small piles<sup>2</sup>. Sixteen bone clusters have been excavated, each representing an episode of butchery and discard and containing the remains of one domestic sheep, or several wild animals or several humans (see, for example, ref. 3).

The carcasses were all butchered using the



Fossil species (left) and rotifera (right). a, *Ceratocystis*, a cornute carpodid; b, *Brachionus* spp., rotifer; c, *Mitrocystella*, a mitrate; d, *Colurella* spp., rotifer; e, *Rhabdopleura*, hemichordate, single individual from a colony; f, *Rotaria* spp., single, individual rotifer from a colony. Scale bars: left-hand side, 1 cm; right-hand side, 100  $\mu$ m.

same procedures. Both human and animal bones have many cut marks in places similar to those seen in bones butchered by modern techniques<sup>2,4</sup>. All the long bones were broken, to extract marrow<sup>2-4</sup>, and the patterns of discard are identical, showing identical treatment of human and animal remains.

If, as Bahn suggests, human bones indicate secondary burial, it logically follows that the Fontbrégoua people hunted, herded and butchered, but did not eat, food animals, and that they gave secondary burial to boars, deer, sheep, roe deer, badgers and marten. At Fontbrégoua the burial hypothesis is not a reasonable alternative and is, in fact, implausible; cannibalism is the only satisfactory explanation.

PAOLA VILLA

University of Colorado Museum,  
Boulder, Colorado 80309-0315, USA

JEAN COURTIN

100 Boulevard de la Libération,  
13004 Marseille, France

BAHN REPLIES — I stated, on the basis of ethnographic data from Australia which had been specifically compared with Villa and Courtin's evidence<sup>5</sup>, that mortuary rituals among some Aborigines can produce the same results as alleged cannibalism.

I would like to make three brief points.

First, the Fontbrégoua human remains are not mixed with animal bones, but treated separately. Second, the limitation of the remains to a brief episode could equally be argued to indicate a mortuary ritual rather than a sudden and short-lived craving for human flesh. Third, one could apply the same logic to both animal and human remains, and assume identical butchery and discard equals consumption in both cases.

At first glance, the last possibility appears to be correct; but whereas there is much evidence throughout history for the consumption of animals by humans, there is virtually no solid, reliable evidence from any period for human cannibalism. Although Fontbrégoua is the best documented case yet published for the existence of prehistoric cannibalism, its separate treatment of human remains, together with the Australian ethnography which can account for every feature in its data, weaken that case considerably.

PAUL G. BAHN

428 Anlaby Road,  
Hull HU3 6QP, UK

1. Bahn, P. G. *Nature* **348**, 395 (1990).
2. Villa, P. et al. *Bull. Soc. Préhist. Française* **82**, 389-421 (1985).
3. Villa, P. et al. *Science* **233**, 431-437 (1986).
4. Villa, P. & Mahieu, E. *J. hum. Evol.* (in the press).
5. Pickering, M. P. *Aust. Archaeol.* **28**, 35-39 (1989).

## Analysis of choroideraemia gene

SIR — Choroideraemia is a heritable X-linked disease that leads to progressive retinal degeneration and blindness. Although this disease is so rare that no accurate data on prevalence are obtainable, choroideraemia is important because it and gyrate atrophy of the retina and choroid are the two hereditary retinal degenerations in the general category of retinitis pigmentosa that can be specifically recognized by the appearance of the retina. The choroideraemia locus has been mapped<sup>1,2</sup> to the q21 band on the X chromosome and DNA clones that span Xq21 deletions in patients with the disease have been isolated. Sequence analysis reveals that these deletions interrupt an open-reading frame of 948 base pairs that could encode a protein of 316 amino acids. RNA transcripts from this open reading frame are absent or structurally altered in patients with choroideraemia, supporting the presumption that it represents the choroideraemia gene. To understand how alterations or the absence of the choroideraemia gene contribute to retinal degeneration its function in eye physiology must be identified. But as Cremers *et al.* point out<sup>1</sup>, neither the nucleotide nor the

predicted amino-acid sequence of the choroideraemia gene have revealed significant homology to gene or protein sequences in the nucleic acid or protein databases (up to August, 1990). Moreover, no topogenic sequences or domains with biological function have so far been identified. We now report the identification of a significant similarity in the amino-terminal region of the putative choroideraemia gene product to a recently described p25A GDP dissociation inhibitor (smg p25A-GDI) (ref. 3), following a search of the updated NBRF protein database.

*In vitro*, p25A-GDI inhibits the rate of GDP release from a ras-like protein, p25A (ref. 3). It has been proposed, as in the case of GAP, IRA1, IRA2 and NF1<sup>4,5</sup>, that smg p25A-GDI could negatively regulate the signalling pathway of p25A by decreasing the intracellular concentration of the active GTP-bound form<sup>6</sup>. But neither the effector function nor the physiological role of smg p25A have been clarified.

An alignment of the regions of smg p25A-GDI and choroideraemia that are similar is shown in the figure. Choroideraemia and smg p25A-GDI are 76 per cent similar over a

46 amino-acid sequence (amino acids 22-67 of choroideraemia and 210-255 of p25A-GDI) with 23 identities and 12 conservative amino-acid residue replacements with no gaps. Particularly striking is the conservation of three prolines at positions 35/223, 39/227, and 47/235 (choroideraemia/p25A-GDI), suggesting a similar secondary structure.

Signal transmission in the retina is mediated by transducin, a trimeric G protein that links photoactivation of rhodopsin to activation of retinal cyclic GMP-phosphodiesterase<sup>7</sup>. Analysis of rod transducin  $\alpha$ -subunit reveals several regions that are similar to the *ras* gene products which correspond to domains involved in guanine nucleotide binding and GTPase activity<sup>8</sup>.

It is, therefore, possible that transducin and the ras-like proteins share related control mechanisms. It is not known whether the similar domain between choroideraemia and p25A-GDI is involved directly in guanine nucleotide metabolism or if it represents a recognition motif for another function. Furthermore, it is noteworthy that Bowes *et al.*<sup>9</sup> have demonstrated that retinal degeneration in the rd mouse model is caused by a defect in the  $\beta$ -subunit of the rod cGMP-phosphodiesterase. So we feel it is likely that other lesions in the visual signalling pathway involving GTP could precipitate retinal degeneration. The homology we present here should help in investigating the function of the choroideraemia gene in retinal disorder.

ERIC FODOR

Departments of Biochemistry and  
Biophysics,

ROBERT T. LEE

JAMES J. O'DONNELL

Department of Ophthalmology,  
Kimura Laboratory of Clinical  
Investigation,  
University of California  
School of Medicine,  
San Francisco,  
California 94143-0730, USA

1. Cremers, F. P. N., van der Pol, D. J. R., van Kerkhoff, L. P. M., Wieringm, B. & Ropers H. -H. *Nature* **347**, 674-677 (1990).
2. Nussbaum, R. L., Lewis, R. A., Leskom, J. G. & Ferrell, R. *Am. J. hum. Genet.* **37**, 473-481 (1985).
3. Matsui, Y. et al. *Molec. cell. Biol.* **10**(8), 4116-4122 (1990).
4. Martin, G. et al. *Cell* **63**, 843-849 (1990).
5. Ballester, R., Sanders, D. A. & McCormick, F. *Cell* **63**, 851-859 (1990).
6. Bourne, H. et al. *Nature* **348**, 125-132 (1990).
7. Yau, K. & Baylor, D. A. *Rev. Neurosci.* **12**, 289-327 (1989).
8. Tanabe, T. et al. *Nature* **315**, 242-245 (1985).
9. Bowes, C. et al. *Nature* **347**, 677-680 (1990).

### Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. They need not arise out of anything published in *Nature*. In any case, priority will be given to letters of fewer than 500 words and five references. □

Chor. 22 KNFLHCLGRYGNTPFLFLYGGGQGGELPQCFCRMCAVFGGIYCLRHVS  
GDI 210 KLYSESLARYGKSPYLYPLVGLGLGLPQGFARLSAIYGGTYMLNKPV

Alignment of the choroideraemia product and smg p25AGDI. II, similar amino acids; I, conservative replacements; \*, proline residues. A Monte-Carlo statistical analysis of this homology was carried out using the EUGENE sequence analysis program (Baylor University) yielding a score of 12, indication a high probability of relatedness.



# Nothing on the rocks

Paul G. Bahn

**Ancient North America: The Archaeology of a Continent.** By Brian M. Fagan. *Thames and Hudson*: 1991. Pp.480. £19.50, \$29.95.

ONE OF Sir Mortimer Wheeler's less perceptive remarks, made to Glyn Daniel, was that American archaeology was "peripheral and of no interest to anyone . . . it's barbaric". Even Gordon Childe wrote that pre-Columbian American archaeology was "outside the mainstream of history".

This kind of attitude still appears inherent in archaeological teaching in the Old World, and notably so in Britain. The undergraduate course I followed almost 20 years ago featured one token lecture on Mesoamerica and one on the Incas; North America was scarcely mentioned. Little has changed since then, although American theoretical musings on the discipline now loom large in present-day courses; and Britain, despite having (almost) the same language as North America, still has only one university post devoted to New World archaeology.

Yet North America has one of the most varied and fascinating archaeological records in the world. Within its wide range of environments, from the Arctic to the desert, it features economies as diverse as whale-hunting and maize farming. It comprises waterlogged sites (especially in Florida, including preserved brains that are yielding ancient DNA), desiccated material galore (in innumerable desert caves and shelters), and frozen remains (both animal and human). There are beautifully made projectile points, bison-drive sites, enormous earthworks, the dramatic pueblo architecture of the southwest, and even pseudo-Mesoamerican cities like Cahokia.

Brian Fagan has done a remarkable job in synthesizing some of the most important items in the vast literature on North American archaeology. *Ancient North America* is more than just a trot through the different regions and periods. He casts a critical eye over the evidence, and amalgamates not only the (often dull) tool typologies but also technology, subsistence and social life. There are useful chapters on the 'discovery' of North America by Europeans, and on the history and development of American archaeological approaches up to and including the current floundering around in search of a specific theoretical goal. In spite of its title, the book also contains a brief survey of some projects in historical archaeology, from shipwrecks to gardens, and from slaves to missions. There is no mention of the thorny topic of reburial of human remains, but it may be no coincidence that this large book contains only one old photo of a human skull.

Although this is a textbook and one, moreover, aimed primarily at North American students, it is highly readable throughout and

will certainly appeal to the nonacademic. Indeed, it will undoubtedly establish itself as the best and most up-to-date treatment of its subject.

Inevitably one has queries and quibbles; the treatment of Monte Verde is a trifle overcautious in view of T. D. Dillehay's recent excellent monograph on the Chilean site; there are some curious omissions such as Wilson Butte (although it is marked on a

ranks among the finest in humanity's heritage.

The greatest irony is that two Texas pictographs appear in full colour on the book's cover to attract the reader. Yet they are not mentioned in the text, which gives only the briefest of nods to Chumash and Fremont rock art, each with a small photograph. One searches the bibliography in vain for the fundamental volumes by K. F. Wellmann or P. Schaafsma; only C. Grant's book on Chumash art is deemed worthy of inclusion.

It is high time that rock art was used as more than a source of pretty pictures to lure the buyer, and accepted with open arms as an integral and crucial part of a region's archaeological record. This has never been more true than today when rock art is entering a new era, as pigment analyses and new dating



Picture by Paul G. Bahn

Petroglyphs at Newspaper Rock, Utah — North America is one of the richest parts of the globe in rock art.

map), or W. T. Mulloy's pioneering work on Pictograph Cave, Montana; and in the historical section it would have been nice to see a summary of the work at the Custer Battlefield, surely one of the most evocative projects of recent years.

But the most serious omission is one that is common to many other books on North American archaeology and, indeed, on other regions of the world: there is an almost total absence of rock art. Yet North America is one of the richest parts of the globe in rock art, both prehistoric and historic. It displays a wide range of forms (pictographs, petroglyphs, geoglyphs, petroforms, engravings) and of motifs (humans, animals, 'scenes', 'abstract' designs) often in stunning locations. Much of it — especially in the southwest and California — is of world class, and

methods are at last providing the means of obtaining more objective data: in North America itself we already have direct dates from Texan pictographs (see *Nature* 348, 710–711; 1990) and cation-ratio dates of desert varnish on petroglyphs of bighorn sheep in California's Coso Range that place the figures in the late Pleistocene.

It is to be hoped that if Fagan's book enjoys the success it deserves, the next edition will accept that rock art merits a far more prominent place, especially as it represents our most vivid and direct clue to what ancient North Americans themselves considered important in the spiritual, symbolic and social aspects of their lives. □

Paul G. Bahn, 428 Anlaby Road, Hull HU3 6QP, UK, is a freelance writer on archaeology.

# Cattle killer

Len Goodwin

**Science and Empire: East Coast Fever in Rhodesia and the Transvaal.** By Paul F. Crane. *Cambridge University Press: 1991. Pp.385. £45, \$65.*

EAST coast fever is caused by *Theileria parva*, a minute tick-borne parasite that enters the T lymphocytes and stimulates them to divide indefinitely, like a lymphoblastic neoplasm. The parasites associate themselves with the Golgi apparatus and divide and multiply with the cells. Some, in the form of minute bars and curved rods, are found in the erythrocytes and infect the brown tick, *Rhipicephalus appendiculatus*, when it feeds. The disease can kill 95 per cent of a herd of cattle in three weeks; it is still the subject of active research and there is as yet no practical method of immunization and no effective treatment.

In 1896, the rinderpest virus swept through southern Africa and killed nearly all the cattle. Less than six years later, after the Boer war and the slow and costly replacement of the animals another, almost invariably fatal disease, African coast fever, broke out in Rhodesia. The infection probably came in an animal or a tick that had travelled by land or sea from Dar es Salaam to Umtali on the Mozambique border. It was at first thought to be a virulent form of the well known 'redwater', caused by *Babesia bigemina*, and to which tolerance develops. But it spread very rapidly, notably through the transport riders, whose huge ox wagons became "abandoned in the veld, abandoned beside the bones of their dead oxen". Confusion was inevitable in a country with two sources of authority that distrusted each other (the South Africa Company and the Colonial Office) and a settler population demanding more and more self government. Rhodes had just died, and for the Chartered Company "to admit the existence of a new cattle disease would have sent down Rhodesian shares".

After months of delay, at a fee with a present-day value of £200,000, the company appointed the famous German bacteriologist, Robert Koch, to advise. Koch had already seen the disease in German East Africa in 1897 and had described the dividing forms of the parasite (Koch's blue bodies) in the lymph nodes. But he had decided that it was a severe form of redwater, carried by the blue tick, *R. decoloratus* and that the small bacillus-like rods he saw in the red cells were immature forms of the redwater parasite. In Bulawayo he realized that it was a new, different disease, characterized not by haematuria, but by anaemia, lymphatic involvement and pulmonary oedema. Although he was unable to transmit the infection by blood inoculation, he thought that he had succeeded in making a protective

serum. This subsequently proved to be useless.

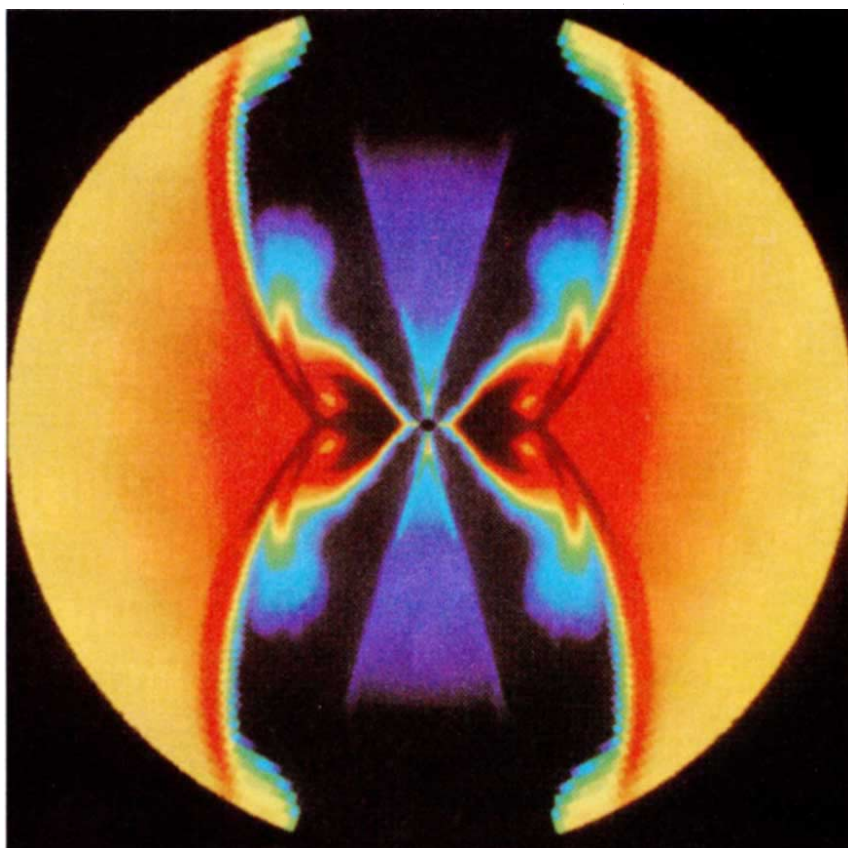
Clarification came from Arnold Theiler, a Swiss veterinarian and Charles Lounsbury, an American entomologist, of the Department of Agriculture of the Transvaal. Theiler showed that the small rods in the red cells were responsible, and Lounsbury proved that the brown, not the blue tick was the vector. Preventive measures were difficult to enforce — the farmers were too poor to accept a slaughter policy — and it took 50 years to bring the disease under control. Although Koch was too self-confident and the Rhodesian veterinarians, Gray and Robertson, were wrong to discount the possibility of a new disease, they deserve some sympathy. Redwater and east coast fever frequently occurred in the same animal, and the very small dots and dashes they peered at, near the limit of resolution of the microscopes of the time, in fact represented four different parasites — redwater (*Babesia*), gall sickness (*Anaplasma*) and two species of *Theileria*, *parva* and *mutans*, sometimes all together in the same erythrocyte.

A key figure behind the scenes was the British Colonial Secretary, Joseph

Chamberlain, who had a deep interest in science and a belief in empire. He was involved in the growing conflict between British and German colonial ambitions in Africa and he resented the appointment of Koch as a consultant. He was convinced that the relief of poverty in Britain depended on the expansion of trade with the colonies and that medicine and science had a crucial role to play. He fostered Kew Gardens, he created the London School of Tropical Medicine and the Royal Society's Malaria and Tsetse Fly Committees, and he believed that civil servants in government should rely upon the scientific advice of experts. We could do with him today.

Paul Crane's method of dealing with the story from the points of view of all the players involved — administrators, politicians, veterinarians, farmers, Africans, cattle, ticks and parasites — makes for a good deal of repetition. But he has tracked down all the documents, from the Colonial Office to the Bulawayo Club, and provides nearly 100 pages of useful notes, comments and references. □

Len Goodwin is at Shepperlands Farm, Park Lane, Finchamstead, Berkshire RG11 4QF, UK.



Computer simulation of a black hole. The spectacular photograph reproduced above represents the output of an extraordinary computer simulation in which  $1.28 \times 10^9$  data points were calculated. The visualization shows the way in which a new supply of low-density matter falls into the dense core of a black hole once the matter in its immediate vicinity has been consumed. The picture comes from *Visualization: The Second Computer Revolution* by Richard Mark Friedhoff, a lavishly illustrated guide to computer graphics and its applications. Published by Freeman; price \$24.95 (pbk).



# A manual for all seasons

D. P. Lane

**Gene Transfer and Expression: A Laboratory Manual.** By M. Krieger. Macmillan/Stockton Press: 1990. Pp.242. £27.95, \$39.

**Protein Methods.** By D. M. Bollag and S. J. Edelstein. Wiley-Liss: 1991. Pp.230. £24.50, \$29.95.

**Molecular Biology: Lab Fax.** Edited by T. A. Brown. Blackwell/Academic: 1991. Pp.322. £21.95, \$49.95.

I AM a great fan of manuals, not just because I have written one myself. At their best, they give real power and inspiration to scientists. One of the most tedious phenomena in science is the 'expert'. This manifests itself in attempts to exclude other scientists from a given area because they are not expert immunologists, molecular biologists, embryologists and so on. We were all beginners once, and a good manual is truly subversive because it breaks down these prejudices and allows scientists to enter new areas with confidence. Manuals are also very cost-effective because they are much less expensive than most 'kits' for molecular or cellular biology. If they prevent a single failed experiment, then they have already paid for themselves. My unit of science is now the postdoc-minute, which is approximately 15 pence in UK currency, so of course one must take account of how long it takes to read the manual as well.

*Gene Transfer and Expression* is excellent. It is written in a wonderfully direct and clear style. It is very cost-effective, as post-doctoral researchers might even read it on holiday. It deals with an expert area that has not been covered in detail before. That is how to get your wretched gene expressed in eukaryotic cells. This is often the rate-limiting step if one wants to sell the gene to a biotechnology company and/or get it to show sufficient biological activity that one can obtain a publishable, perhaps even meaningful, result.

I particularly enjoyed the introductory chapters on the control of eukaryotic gene expression. They contain just the right amount of detail to allow the reader to make choices of host and vector. The protocols themselves, the heart of any manual, are also clear and direct. Their personal touch gives one hope that the author really knows they will work. What also shines through is the author's honesty. When he does not understand something, he says so rather than let it hang in the air. He is also happy to be specific about which machine and which chemical to buy. This is very helpful when starting out on a new technique. The book is very comprehensive and covers the whole area of eukaryotic expression from retroviral vec-

tors to expression cloning.

*Protein Methods* is less satisfying. It deals with two major topics; preparation of protein extracts and gel electrophoresis of protein samples. In a way, this is not a bad mix of topics and the manual contains a lot of sensible points about the preparation of cell extracts. But the protocols are often sketchy or incomplete, and frequently refer the user to other manuals for a given step, which is very irritating. The section on gel electrophoresis, although reasonably comprehensive, does not really add anything to the many other texts that cover this area.

*Molecular Biology: Lab Fax* is not a manual, more a gigantic mutant appendix. It is full of the sort of information one finds in

the better catalogues from molecular biology, radiochemical and chemical companies. Genotypes of bacteria, restriction sites, enzyme buffers, decay curves for  $^{125}\text{I}$  are all here. The book scores in my view because it has collected all these things together in one place, and although it will probably only inspire train-spotters, I think it is well worth getting.

Soon, I suppose, we will have manuals for every method and probably be updated continually by computer mail. Perhaps it is time to write a manual about thinking. □

D. P. Lane, Cancer Research Campaign Laboratories, Medical Sciences Institute, Dundee University, Dundee DD1 4HN, UK.

## Meat and veg

May Berenbaum

**Plant-Animal Interactions: Evolutionary Ecology in Tropical and Temperate Regions.** Edited by P. W. Price, T. M. Lewinsohn, G. W. Fernandes and W. W. Benson. Wiley: 1991. Pp.639. \$125, £97.35.

LONG before it was politically fashionable, biologists have had an appreciation for the tropics, not only for the breathtaking diversity of the flora and fauna, but also for the unique insights afforded by that diversity into the evolutionary processes influencing life on Earth. Over 100 years ago, A. R. Wallace wrote

To the student of nature . . . the tropics will ever be of surpassing interest whether for the variety of forms and structures which it presents, for the boundless energy with which the life of plants is therein manifested, or for the help which it gives us in our search after the laws which have determined the production of such infinitely varied organisms.

Wallace would no doubt have approved of this latest volume. Publishing symposium proceedings on herbivory of various descriptions has become common practice of late, but *Plant-Animal Interactions* distinguishes itself from the crowd by both its emphasis and its scope. The outcome of an international symposium conducted in Campinas, Brazil, in 1988, this book is refreshingly distinctive in its emphasis on tropical plant-animal interactions.

Although the editors indicate in the preface that the volume is not intended to be a "compendium of research on plant-animal interactions", they have nonetheless managed to pack an extraordinary amount of diversity between its covers. The majority of contributions focus on antagonistic interactions, but mutualistic interactions merit a section of their own. It is not surprising that plant-butterfly interactions are the focus of five chapters — tropical butterflies have attracted the attention of evolutionary ecologists ever since Henry Bates first speculated

about mimicry almost 130 years ago. But a rich diversity of nonlepidopteran herbivores are represented; there are chapters on two-legged (T. H. Fleming on frugivorous birds), four-legged (S. J. McNaughton on mammals), six-legged (nonlepidopterous insects of all descriptions) and even eight-legged (A. J. Heyneman *et al.* on hummingbird flower mites) herbivores. Although the majority of chapters describe basic research, at least three (M. Kogan, M. A. Altieri and M. A. Garcia) focus on agroecology. Even chapter structure varies; some chapters are strictly literature surveys, other are detailed experimental studies, and still others are summaries of many decades of research. Overall, the taxonomic, ecological and stylistic diversity provide for an endlessly entertaining and intellectually challenging read. The editors provide an excellent historical introduction to the book as a whole, as well as cogent and informative introductions to each of the six sections, so the book is thematically and stylistically cohesive.

If this collection has a weakness, it is the inclusion of chapters that focus exclusively or extensively on temperate herbivory. Individually, these contributions are of uniformly high quality; however, they seem out of place in this volume, the main emphasis of which is on tropical herbivores.

Mary Evans

IMAGE  
UNAVAILABLE  
FOR COPYRIGHT  
REASONS

Alfred Russel Wallace, 1823-1913.

The study of tropical interactions is no less critical to the development of ecological thought and evolutionary theory today than it was a century ago, and *Plant-Animal Interactions* is valuable for its intellectual content. Yet even though they are now rigorously quantified and statistically analysed, tropical interactions are not one bit less fascinating to read about today than they were when Wallace admired "that rich variety of colour, and that nicely balanced harmony of relations which delight and astonish us in the animal productions of all tropical countries." □

May Berenbaum is in the Department of Entomology, University of Illinois, 320 Morrill Hall, Urbana, Illinois 61801, USA.

## Firm foundations

W. F. Bynum

**Partners in Science: Foundations and Natural Scientists, 1890-1945.** By Robert E. Kohler. University of Chicago Press: 1991. Pp.480. \$34.95, £27.95.

LIKE the Renaissance artist, or eighteenth-century man of letters, the modern scientist needs patrons. The Germans secured state patronage from the middle of the nineteenth century; central government in Britain began — somewhat reluctantly — to support science and scientists from the early decades of the present century; in the United States, the Second World War was the watershed,

logy could be described as a recognized branch of the history of science.

In *Partners in Science*, Robert Kohler has set new standards in the field and although his will inevitably not be the last word, future accounts will have to take his version of the story on board.

In crude outline, the story is simple. Andrew Carnegie (1835-1918), a Scottish-born immigrant to the United States, and John D. Rockefeller (1839-1937), a native of New York, made gigantic fortunes in steel and oil, respectively. From a variety of complex motives, each devoted a sizeable portion of his fortune to philanthropy. The sums involved were so great that no one person could possibly have overseen their reasonable dispersal, so both men gathered around them advisers, several of whom considered scientific research as an appetizing item on the philanthropic menu. Initially, the format was the institutional one common in nineteenth-century Germany and given worldwide currency by the establishment of the Institut Pasteur in Paris. The Rockefeller Institute (1901) in New York and the Carnegie Institution of Washington (1902) were simply American varieties of a European holotype.

Gradually, however, a much more complicated pattern of science funding evolved, especially through the Rockefeller Foundation and its various agencies, such as the General Education Board and the International Education Board. Although Rockefeller, *père et fils*, took an interest in things, a good deal of policy devolved on advisers and

tists. The boring evaluation of grant and fellowship requests to individual scientists was farmed out to the National Research Council, to which both Carnegie and Rockefeller contributed, leaving headquarters to deal with larger matters.

After the First World War, these matters were often bricks, mortar and equipment, as large sums were given to institutions such as Caltech, Princeton and Harvard in the United States, and, in Europe, establishments in Edinburgh, Göttingen and Copenhagen. The world was not exactly the Rockefeller oyster — Eastern Europe and the Mediterranean countries did not look very promising — but foundation officials refused to be confined to national boundaries in their search for scientific talent. At the same time, the general policy of requiring recipient institutions to match Rockefeller funds limited the number of participants.

Kohler dates a third phase of Rockefeller largesse to the late 1920s, when the foundation was reorganized administratively and when, in 1932, Warren Weaver arrived from Wisconsin to take charge of the grants programme in the natural sciences. He favoured the project grant in such areas as general physiology and what eventually became known as molecular biology. A mathematical physicist himself, Weaver is best remembered for encouraging physical scientists to study what he called 'vital processes'. More than any other of the Rockefeller philanthropists, Weaver became an active partner in the scientific enterprise.

Kohler's book reminds us yet again how rich an archival legacy Rockefeller and his employees left behind, through letters, office memoranda and diaries. These allow for the detailed investigation of policy and personality, and demonstrate how important the latter was. Thinking big paid dividends; so did working in the interstices of established disciplines.

What counted for little in all this was what has become the hallmark of science in the past 40 years: the peer review. Of course, Rockefeller's men took advice, but they did so informally, were suspicious of scientific trustees, had no formal application forms and sought above all independence of judgement and action. They also made mistakes, but, on balance, the record of the foundations stands up to scrutiny. Without them, science in the first half of this century would have been the poorer — and not just financially. In developing partnerships with scientists, they went some way towards erasing Dr Johnson's definition of a patron as "commonly a wretch who supports with insolence, and is paid with flattery". Because Kohler ends his book in 1945, he is spared the task of deciding whether the postwar system of state patronage of science is johnsonian. □

W. F. Bynum is at The Wellcome Institute for the History of Medicine, 183 Euston Road, London NW1 2BN, UK.

## IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Patrons of science — Andrew Carnegie (left) and John D. Rockefeller (right).

after which federal patronage was the most important source of external funding. By then, however, the best American science had become world class, nurtured by private philanthropic foundations, of which the Carnegie Corporation was important, and the Rockefeller Foundation fundamental. This is a phenomenon that was reflected on at length by several of the key participants and has since been analysed by a number of historians, so many in fact that philanthropo-

paid officials who, in the time-honoured way, filtered what reached Rockefeller and his Board of Trustees. In the period before the First World War, a key issue was the active versus the passive foundation: whether foundation officials should identify attractive research areas or simply provide support for the best people who happened to come along. Training programmes were also contentious, because routine teaching seemed the bane of many academic scien-



# Comparative methods for explaining adaptations

Paul H. Harvey & Andy Purvis

The ways that taxonomic differences in morphology, behaviour or life history are related to each other and to differences in lifestyle have been used regularly to test ideas about the selective forces involved in their evolution. Such comparative tests have been transformed recently by using increased statistical rigour. The realization that the statistical model against which comparisons are made is a model of how evolution proceeds, forms the basis of a new generation of comparative tests that are grounded properly on phylogenetic reconstruction.

COMPARISON among species is the most commonly used technique for examining how organisms are adapted to their environments. If we compare snowy owls with tawny owls and polar bears with brown bears, we note that the different species often match the colour of their habitats. This leads to the reasonable conjecture that natural selection against being conspicuous has produced the colour differences. We even make such comparisons with species that do not exist. If we were to ask why giraffes have long necks, we might answer that short-necked giraffes would lose in feeding competition against their long-necked relatives, who could browse from the tallest trees. These uses of what is now called 'the comparative method' provided the evidence for many central arguments in Darwin's *The Origin of Species*<sup>1</sup> and *The Descent of Man*<sup>2</sup>, and are still employed by most evolutionary biologists in their day-to-day research.

One reason that the comparative method has proved so important is that many evolutionary explanations are not open to experimentation. We cannot physically rerun the evolutionary sequences that produced snowy owls, polar bears or white rabbits from their tawny ancestors. When evolutionary experiments are possible, for example by breeding from white mutants, the time and effort involved limits us to a few case studies. Even then the results are of limited value for understanding evolutionary processes in natural populations, and less for generalizing results to other species. Fortunately, evolution on Earth has provided countless natural experiments whose results can be used with great profit. Take the question of why male chimpanzees have much larger testes than male gorillas. Perhaps, it has been suggested<sup>3-5</sup>, this difference has to do with the fact that female chimpanzees mate with several males, whereas female gorillas do not. One way that natural selection can increase a male chimpanzee's chances of fathering a female's offspring is to produce more tickets in the lottery of life by generating more sperm per ejaculate. If that explanation is correct, other primate species with promiscuous females should also have large testes and produce more sperm per ejaculate. This prediction and others concerning sperm motility have been supported by comparative studies for primates<sup>6-8</sup>, other mammals<sup>9</sup>, and even birds<sup>10,11</sup>.

The breadth of comparative studies is impressive. They have continued to provide new insights into behaviour, genetics, physiology, development, ecology and morphology<sup>12-21</sup>. And they are not limited in their scope to particular taxonomic groups, having been applied to animals, plants, viruses, fungi and bacteria<sup>22-26</sup>. But over the past decade a new rigour has begun to pervade the comparative approach to studying adaptation. Techniques have been developed to deal with two related problems faced by comparative studies: evolutionary history and alternative explanations.

Compare the mating systems of birds and mammals. Most bird species breed as monogamous pairs<sup>27</sup>, whereas most mammals are polygynous<sup>28</sup>: one or a few males defend a group of

breeding females. What is the reason for such variation in mating systems? It has been common practice in such cases to perform straightforward statistical analysis counting species as independent data points<sup>26,29</sup>. If we lump all the bird and mammal species together, egg-laying or the presence of feathers will be found in most species that are monogamous, whereas viviparity (the bearing of live young) and the presence of fur are found with polygyny. Indeed, any characteristic distinguishing birds from mammals will turn out to be associated with differences in mating systems.

A satisfactory comparative test can be performed only when we use information from polygynous birds and monogamous mammals. Rather than simply comparing all extant species, we need to locate instances in which one or the other mating system has evolved, and examine which other characters changed at the same time. Both of the mating systems have evolved several times in birds and mammals; it is just that a different one has come to predominate in each group. If we had perfect phylogenetic trees for birds and mammals, and knew the characteristics of all the ancestors, we could seek independent evolutionary origins of both monogamy and polygyny. It might be, for example, that whenever monogamy evolved it was associated with territoriality, but when territoriality broke down then polygyny arose. By seeking independent evolutionary origins of characteristics, we can identify independent points for statistical analysis and thereby control for confounding variables like fur and feathers that are associated with phylogenetic differences<sup>30</sup>.

The most satisfactory comparative analyses are based on accurately reconstructed phylogenies. Unfortunately, we do not have perfect knowledge of evolutionary trees or of ancestral character states. Instead, phylogenies have to be reconstructed using models of how we think evolution works<sup>31</sup>. A later section describes how different models applied to the 'same' contemporary species data give different estimates of ancestral character states and different answers to comparative questions. Before considering different models of evolutionary change, we summarize why material from contemporary species contains information about the evolutionary relationships among them.

## Why are closely related species similar?

Species values do not provide independent points for comparative analyses because species share characteristics through descent from common ancestors<sup>32</sup>. And the more closely related that species are, the more similar they tend to be. This tendency has often been termed 'phylogenetic inertia'. What causes it? It is possible to identify three distinct possibilities that can be termed 'phylogenetic niche conservatism', 'phylogenetic time lags', and 'phenotype-dependent responses to selection'<sup>26,33</sup>.

**Phylogenetic niche conservatism.** Those species that move into new or vacant niches are likely to have occupied similar niches elsewhere<sup>34</sup>. For example, if a freshwater lake were to be formed following a landslide, it would probably be invaded by aquatic

species from nearby rather than terrestrial organisms, and those aquatic organisms would probably have come from freshwater rather than marine habitats. Furthermore, although invading species might evolve to fit niches that differed slightly from their ancestral niches, opportunity for niche expansion would be limited by competition from other species invading the new habitat. On the rare occasions that such competitors are absent, adaptive radiation occurs as with Darwin's finches<sup>35</sup>.

**Time lags.** It takes time for organisms to evolve adaptations for new lifestyles and, in the extreme, there may not be appropriate genetic variance for selection to act upon. For example, attempts to select for biased sex ratio in *Drosophila* have frequently failed<sup>36</sup>. Indeed, turning to vertebrates, the financial reward for producing a breed of chickens which laid mainly female eggs would be considerable.

**Phenotype-dependent responses to selection.** Organisms with similar phenotypes may respond in similar ways to the same selective pressures, whereas organisms with different phenotypes may respond differently. Changing pressures of sexual selection have resulted in appropriate adjustments in weaponry used in inter-male combat: antlers for deer<sup>37</sup>, canine teeth for primates<sup>38</sup>, and horns for antelopes<sup>39</sup>. Antelopes have not evolved antlers. Indeed, the same selective pressure can even produce evolutionary change in opposite directions in different taxa. In response to increased predator pressure, small mammals may become smaller, and therefore be able to take more effective refuge at the tops of trees or down burrows, whereas large species may become larger, thereby being better able to fight off predators<sup>40</sup>. Similarly, in response to predators, distasteful lepidopteran larvae may become brightly coloured, whereas palatable species become cryptic<sup>41</sup>. With increased intraspecific competition for food, species that defend resource patches may evolve larger size when size is correlated with success at fighting with conspecifics, whereas species living on undefendable resources may become smaller. For example, smaller grazing mammals can persist on more closely cropped swards<sup>40</sup>.

### Criteria for comparison and reconstruction

Comparative tests that are based on incorrectly reconstructed phylogenetic trees may either overestimate or underestimate the number of independent evolutionary events. If random errors are made in phylogeny reconstruction, the amount of evolutionary change is likely to be overestimated. For example, if one error was made in the phylogeny of mammals so that chimpanzees were placed as the closest living relatives of kangaroos, we should infer additional independent evolutionary origins of placentation, encephalization and many other characters. By contrast, if a phylogeny is reconstructed on the basis of characters used in a comparative test, the number of character-state transitions is likely to be underestimated. For example, if palatability of caterpillars was the sole criterion for phylogenetic tree reconstruction among the lepidoptera, then all palatable caterpillars would be placed together in one monophyletic group (that is, the species emanating from a particular common ancestor) and all the unpalatable species in another; any comparative test concerning the evolution of palatability or unpalatability within the lepidoptera would be based on a sample size of one.

As with the reconstruction of ancestral character states, phylogenetic tree reconstruction also depends on having a model of evolution in mind<sup>31,42</sup>. The most plausible tree is chosen according to some set criterion, such as the minimum amount of total evolutionary change in the tree, and the trees for the different characters used in phylogenetic reconstruction are compared to produce a consensus tree. The criteria for defining the 'most plausible tree' and a 'consensus' tree must, inevitably, be based on some idea of the way evolution works.

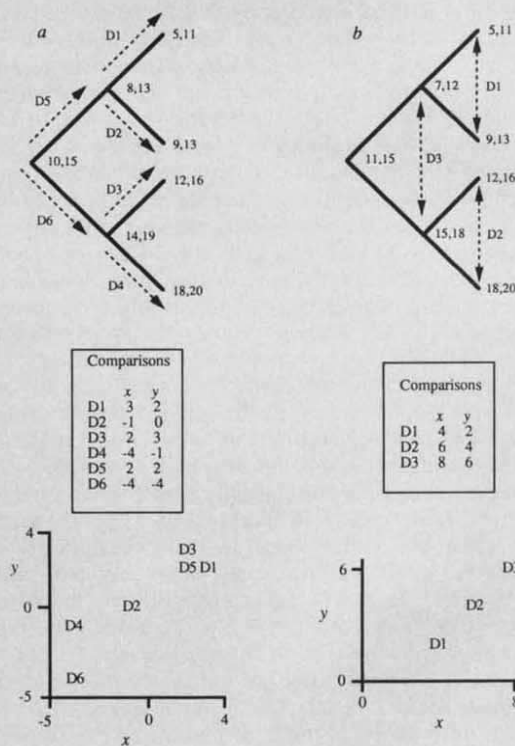
### Types of comparative test

Once a phylogenetic tree has been chosen and attributes of the living species have been recorded, which comparisons should

be made to test for correlated evolution? Many methods have been proposed, some of which are variants on others, but almost all entail making different comparisons<sup>26,30,32,34,43-51</sup>. Most suggestions have proved inadequate for general purposes because they discard or ignore some of the data, make unacceptably unrealistic assumptions about evolutionary processes, or pre-judge the contributions of various factors to phylogenetically correlated similarity<sup>26,52</sup>. Nevertheless, many methods have provided invaluable stepping stones for the development of more acceptable tests. It seems clear now that two basic approaches can usefully be adopted (Box 1). Directional methods compare ancestors with their descendants, whereas nondirectional methods can be thought of as comparing the immediate descendants of a common ancestor with each other; they make comparisons within evolutionary radiations.

In each case, we simply tally the comparisons and perform a significance test on the result. Each test has as many statistical degrees of freedom as there are comparisons. However, there

**BOX 1. Types of comparative test: directional and non-directional analyses**



A bifurcating phylogenetic tree is shown with four extant species. Dotted, arrowed lines denote the taxa compared, and pairs of numbers at the tips and nodes of the tree give states of characters *x* and *y*. In case *a*, ancestral character states are known, allowing directional comparisons to be made between ancestral and daughter taxa. In case *b*, ancestral character states are not known but are estimated as the average of daughter taxon values. Differences (*D*) in character state values between the immediate descendants of a common ancestor are made in *nondirectional comparisons*, with the sign of the *y* comparison being determined by making the *x* difference positive. Extra degrees of freedom are available when character states are known, so that six directional and three nondirectional comparisons are possible in the above example. To compare ancestral and daughter taxa values when the former are calculated from the latter would introduce dependence into the comparisons. Ancestral character states are estimated for nondirectional comparisons by assuming some particular model of evolution: in the above example the model is one of brownian motion<sup>47</sup>. Some other models are considered in Box 2.



are more directional than nondirectional comparisons: for a bifurcating tree with  $n$  extant species, there are  $2n - 2$  directional comparisons but only  $n - 1$  nondirectional comparisons. This difference arises because in the directional case it is assumed that ancestral character states, represented by taxa at nodes of the phylogenetic tree, are known independently of those for living species. With  $n$  species there are  $n - 1$  nodes, making a total of  $2n - 1$  values, giving  $2n - 2$  possible degrees of freedom. By contrast, with nondirectional studies nodal values are estimated from those of the living species, and with  $n$  species there can be only  $n - 1$  degrees of freedom.

If ancestral character states are not known but are calculated in part or whole from daughter taxa, then comparisons between ancestors and their descendants are not valid because the ancestral value depends in part on the value of each daughter taxon. Furthermore, because the ancestral character states are likely to have been estimated from all immediate daughter taxa, comparisons between an ancestor and different daughter taxa will not be independent of each other. For example, if an ancestral character state is estimated as the average of two daughter values  $(a + b)/2$ , then the difference between one daughter and the ancestor,  $a - (a + b)/2 = a/2 - b/2$ , will be identical in magnitude but opposite in sign from the difference between the other daughter and the ancestor,  $b - (a + b)/2 = b/2 - a/2$ . These problems should be taken into account in directional studies which compare reconstructed ancestral character states with values for daughter taxa<sup>15,22,26,50,53-59</sup>. Appropriate nondirectional comparisons can avoid the problem of dependence because putative ancestors are not compared with daughter taxa.

Directional analyses can often answer different questions from those addressed by nondirectional comparisons. For example, by comparing ancestors with their descendants, Cope formulated his law that the members of a lineage get larger through evolutionary time<sup>60</sup>. Examples of parallel evolution as illustrated by Cope's law will not be revealed by making nondirectional comparisons among sister taxa—such comparisons tell us nothing about the temporal direction of change (Fig. 1). Directional comparisons may also help us to determine cause and effect in cases where two traits have coevolved. For example, brightly coloured butterfly larvae are often distasteful. Presumably bright coloration acts as a warning to predators and evolved in lineages in which the species were already distasteful<sup>61</sup>. Suitable directional comparisons would be predicted to demonstrate this pattern, rather than one of distastefulness evolving in lineages with brightly coloured individuals.

Directional comparisons have great potential for testing ideas about adaptation. In principle, they can tell us everything that nondirectional comparisons can, and more. If the data for extant species and their ancestors are available, directional comparisons should be made. But data on the ancestors are rarely available: we do not yet know whether the ancestors of particular butterflies were distasteful or brightly coloured. The best that can be done is to use what is considered an appropriate model of evolution to estimate plausible ancestral character states from information on the branching structure of the phylogenetic tree and from measures on extant species.

### Evolutionary models and ancestral states

The question of how evolution proceeds is a contentious one. Indeed, different traits may show different modes of evolutionary change within a single lineage. Furthermore, rates of change or probabilities of change may vary among lineages. Faced with so many scenarios, any of which might be correct in a given case, how should ancestral character states be estimated?

One attractive possibility is to seek the maximum likelihood ancestral character states under different models of evolutionary change. But maximum likelihood solutions are notoriously difficult to achieve<sup>62,63</sup>. Instead, it is more usual to attempt to reconstruct ancestral character states so that the total evolutionary change in the whole tree is minimized. Outgroup comparison

TABLE 1 Lekking and size dimorphism in birds

|             | Across species |               | Independent events |               |
|-------------|----------------|---------------|--------------------|---------------|
|             | Size dimorphic | Not dimorphic | Size dimorphic     | Not dimorphic |
| Lekking     | 69             | 18            | 11                 | 9             |
| Non-lekking | 13             | 13            | 6                  | 9             |

The strong cross-species association between mating system and size dimorphism is not found when independent evolutionary events are considered instead (after Höglund<sup>68</sup>).

has often been advocated as a method for reconstructing ancestral character states for dichotomously varying characters under the assumption of parsimony or minimum change<sup>30,64</sup>. Futuyma<sup>65</sup> gives an example: anterior legs of the butterfly families Nymphalidae and Danaidae are reduced, whereas those of Papilionidae and Pieridae are functional. Moths, the outgroup to the butterflies (that is, the most closely related species or group of species to the monophyletic butterflies), have functional anterior legs which might be accepted as the hypothesized ancestral state. Unfortunately, outgroup comparison provides local but not necessarily global parsimony solutions<sup>66</sup>. For example, if the next outgroup to the moths had reduced anterior legs, alternative parsimonious sets of ancestral states would be possible: two independent origins of functional anterior legs (in the butterflies and the moths) from ancestors with nonfunctional anterior legs, or two independent origins of nonfunctional anterior legs (in the butterflies and the new outgroup) from ancestors with functional anterior legs. In fact, as Futuyma<sup>65</sup> points out, likely outgroups to the butterflies and moths have functional anterior legs. Algorithms, simple rules, and computer programs are available to provide parsimony solutions for the smallest number of character-state transitions under particular rules of change (reviewed in ref. 67).

When ancestral character states for pairs of dichotomously varying traits have been estimated, suitable non-directional comparisons can be made to test for correlated evolution. Ridley's method<sup>30</sup> compares only those taxa at the ends of branches along which some evolutionary change is believed to have occurred. End states for all such branches are tallied and cast into a two-by-two contingency table. Methods such as this can give very different results from those in which species are considered as independent points. For example, Höglund<sup>68</sup> examined the relationship between mating system and sexual size dimorphism among 113 bird species. In his sample, size dimorphism was significantly more common among lekking than non-lekking

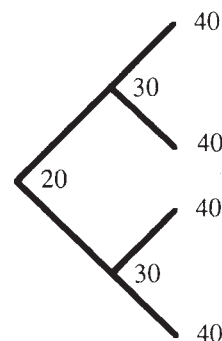


FIG. 1 A measure of body size is given for each extant species and the ancestors at nodes of the phylogenetic tree. By comparing ancestors and descendants in a directional analysis, Cope's law of increasing size through evolutionary time is evident. But the three comparisons made in a nondirectional analysis on these data (by comparing sister taxa in this case, as in Box 1) would reveal no differences among the taxa being compared.

species. But because both traits show strong phylogenetic associations, Höglund could find no evidence for correlated evolution between the two traits (Table 1).

Many characters, such as body weight, vary more or less continuously both across and within extant species. As Box 2 shows, estimated ancestral character states and the results of comparative analyses for continuous variables differ according to the model of evolution that is assumed.

If we have no faith in any of the available models evolutionary, but are willing to accept the structure of the phylogenetic tree at lower levels, an alternative procedure can be used to detect correlated evolutionary change between traits<sup>47,69,70,79</sup>. Comparisons are made within pairs of related species. Thus, the differences between pairs of apes, lemurs and macaques have evolved independently of each other. Such differences are non-directional, comparing descendants with descendants, but because no ancestors are compared, no model of evolution need be assumed to estimate them. The method has a much lower power than those discussed earlier—only  $n/2$  comparisons can be made, because each uses two species' values—and only nonparametric statistics should be used because the comparisons will not have equal variances. Nevertheless, the method can be very useful, particularly when the higher-level phylogeny is not known. An example of this approach is Møller and Birkhead's study of copulation in birds<sup>71</sup>. The authors proposed that colonial birds have a higher extra-pair copulation rate than

solitary breeders, and predicted that paired males in colonial species should copulate more frequently so that their mates' offspring are less likely to be fathered by other males. Comparisons between solitary and colonial populations of the same species, and between congeneric species differing in their social dispersion, gave strong support to the prediction.

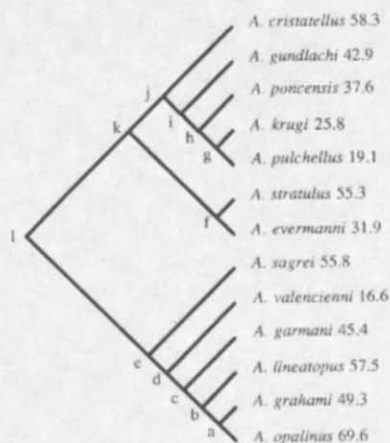
### Statistical considerations

The use of many standard parametric statistical techniques on the comparisons requires that each comparison be independent of every other, and that there be homogeneity of variance throughout the sample.

If the model of evolution is in error then nondirectional comparisons lose some independence. This problem has been considered by Martins and Garland<sup>72</sup> who report a simulation study which compared the type I and type II error rates of various methods under models of evolutionary change where character transition occurs only at the nodes of trees and those where it may occur at any point in time along branches (types of punctuational and gradual evolution, respectively). Unsurprisingly, the method that performed best was the one under whose assumptions a set of data was generated. More interestingly, a range of punctuational methods performed fairly well under a gradual evolutionary regime, and vice versa. In all cases the worst method of all, with highest type I and type II error rates, was a simple cross-species analysis.

#### BOX 2. Results of comparative tests depend on models of evolutionary change

The figure shows the phylogenetic tree for a group of *Anolis* lizards, and a measure of running activity for the extant species<sup>55,56</sup>. This information together with branch lengths (also from refs 55, 56) is used to estimate the character states at the nodes of the tree labelled a to l. These estimates are no more than that; they are inferences made at an intermediate stage of the statistical analysis, and should not be used outside that context.



One possible evolutionary scenario is that change may occur in each time interval along the branches of the tree. A brownian motion model of evolution<sup>47</sup> assumes that the direction of change at each time interval in each branch is random. If the branch lengths leading to two extant sister species are the same, their direct common ancestor may be estimated as the mean of their two values (this procedure does not provide the maximum likelihood estimate). Otherwise the ancestor is estimated as being more like the daughter taxon at the end of the shorter branch: the daughter that has been randomly walking for the shorter length of time is likely to be more similar to the ancestor. These estimates are made with error, which must be taken into account when we use them to estimate nodes nearer the root of the phylogeny. We can move through the tree from tips to root in like fashion, estimating ancestors from daughter values, appropriately weighted by branch lengths (the procedure is described in detail and justified elsewhere<sup>47</sup>); these estimates are shown below under model 1.

Alternatively, change may be more punctuational, occurring only at nodes of the tree. If change occurs in both daughter branches derived from a node and the direction of change in each is random, an estimate for the character state in the immediate common ancestor of two extant sister species is their average. The ancestral reconstructions estimated from this model are shown under model 2. They again incorporate error adjustments. Note that the estimates produced under this model are the same as those produced under the brownian motion model (model 1) if it is assumed that all branch lengths are equal.

A third approach is to compute the ancestral values using a parsimony assumption. The reconstruction used as model 3 minimizes the squared changes summed along all branches of the tree<sup>54</sup>. Such a method results in the comparisons not being strictly independent because the procedure makes use of all the species values to estimate each ancestor.

| Ancestral node estimate | Model 1 | Model 2 | Model 3 |
|-------------------------|---------|---------|---------|
| a                       | 59.5    | 59.5    | 57.4    |
| b                       | 58.6    | 58.3    | 53.2    |
| c                       | 53.6    | 50.4    | 44.8    |
| d                       | 42.5    | 29.5    | 35.8    |
| e                       | 47.8    | 45.8    | 46.0    |
| f                       | 43.6    | 43.6    | 44.6    |
| g                       | 22.5    | 22.5    | 26.9    |
| h                       | 28.2    | 31.5    | 35.7    |
| i                       | 33.0    | 38.5    | 42.6    |
| j                       | 40.7    | 50.7    | 49.2    |
| k                       | 41.9    | 47.0    | 46.7    |
| l                       | 45.3    | 46.4    | 46.4    |

When suitable nondirectional comparative analyses are performed to test whether evolutionary changes in the measure of running activity are significantly correlated with evolutionary changes in relative foreleg length (foreleg length corrected for body size), the result depends on the model of evolution used. For the three models given above, the *F*-values obtained from regression through the origin over the 12 possible comparisons are, respectively, 7.14 ( $P < 0.025$ ), 3.24 ( $P > 0.10$ ) and 2.82 ( $P > 0.10$ ). It is important when performing such tests to check that relationships between the values of the original variables do not have systematic departures from linearity<sup>26</sup>; appropriate transformations of the original variables can usually make such relationships linear. None of the relationships investigated here had significant curvilinear components.



Provided that degrees of freedom are estimated correctly, nonparametric statistics can be used<sup>20,73,74</sup>. Parametric tests, although more powerful, frequently make the additional assumption that all points in the sample have the same expected variance. This requirement can be met by scaling each comparison by the expected variance from the model of evolution being applied in any particular case. Different models of evolution require different scaling functions. It is always desirable to check for homogeneity of variance in comparisons before they are used in parametric tests.

Testing hypotheses for correlated evolution between characters involves comparing evolutionary changes in one character against changes in the other. Clearly, if a comparison is being made between the states of characters  $x$  and  $y$  for two species, say  $a$  and  $b$ , then if difference in  $x$  is calculated as  $x_a - x_b$ , the difference in  $y$  should be calculated in the same direction (that is,  $y_a - y_b$  not as  $y_b - y_a$ ). It can be convenient to choose the direction of comparison so that the result for one of the variables being compared is always positive (this was done for  $x$  in Box 1). Whether that procedure is adopted or not, in the absence of correlated evolution, the slope of a regression forced through the origin between the two sets of contrasts should not differ significantly from zero<sup>34</sup>. When correlated evolution is present, it may be necessary to fit a nonlinear regression or to transform the data to linearize the relationship<sup>26,75</sup>.

### Problems with using real data

Reconstructed phylogenies are often inaccurate or, at best, coarse descriptions of evolutionary history. In particular, even when derived from a reasonable model of evolutionary change, several dichotomous branching events may be confounded into a single polytomy (such as when three species are placed in a single genus), branch lengths may be unspecified, and the branching structure of phylogenetic trees may be incorrect. Unless these problems are recognized and dealt with explicitly, misleading conclusions can be drawn from comparative analyses. Several techniques have recently been developed which, when taken together, allow reasonably reliable comparative statements to be made within the limits dictated by such imperfect data.

In the absence of more reliable information, evolutionary classifications (which arrange species into hierarchically defined taxa such as genera, families and orders) are used to describe the approximate phylogenetic trees that form the basis of comparative studies. Such classifications are often incompletely resolved, with more than two subtaxa frequently contributing to a single branching event. For example, if there are three species in a genus, we might reasonably assume that the genus represents a monophyletic group, with two of the species sharing a more recent common ancestor than the third<sup>34,76</sup>. Methods have been developed to estimate ancestral character states with polytomous cladograms<sup>76</sup>, and various linear contrast constructions have been suggested which reduce such multiple branching patterns to a single comparison for use in nondirectional comparative analyses<sup>26,34</sup>.

Branch lengths are becoming better known, partly through the use of molecular divergence data. But when phylogenies are used which do not specify branch lengths, misleading results may be obtained, even under the correct model of evolution. For example, two of the sets of ancestral character states in Box 2 were estimated under an evolutionary model which assumed that change occurred only at branch points of the phylogenetic tree. Under such a model, differences in branch lengths become irrelevant because they are ignored. In fact, this is equivalent to assuming that all branch lengths are equal. Therefore, just as the minimum-evolution 'punctuational' model indicated non-significance, so would a minimum-evolution 'gradual' model that assumes equal branch lengths. When branch lengths are not known, it may be possible to improve on the assumption of equal lengths if tree structure is taken into account. For example,

if two genera have different numbers of species in them, we might assume that the one with more species is older.

The problem which has received least attention is incorrect tree structure. If alternative trees are likely, and they usually are<sup>47,77</sup>, it is fairly straightforward to search for correlated evolution of characters in the different trees. An example is given by Björklund's analysis of sexual dimorphism in 21 cardueline finch species (see ref. 71): each of five cladograms constructed using different tree-building criteria provided evidence for significantly correlated evolution between male and female tarsus length, and between male and female bill depth. But sexual dimorphism in the tarsus and the beak coevolved significantly in just two of the five trees. Choice of the correct tree in the latter case would be crucial for deciding whether correlated evolution had occurred. Such sensitivity analyses should become commonplace in comparative studies.

### The comparative method now and in the future

The choice of evolutionary model used for comparative analyses is currently more often a reflection of its analytical and statistical tractability than its biological realism; hence the current popularity of brownian motion models of evolution as a basis for comparative tests (no evolutionary biologist actually believes that brownian motion provides a realistic model of evolution). The robustness of the comparative method to violations of the assumptions made by particular models is often not known. Simulation studies can help us to identify the assumptions to which the methods are most sensitive<sup>72</sup>.

The first comparative statistical tests to incorporate phylogenetic information were published 14 years ago<sup>43</sup>. They were criticized correctly as being procrustean<sup>78</sup>. Now we can make independent comparisons of both discretely and continuously varying characters under a wide range of evolutionary models for bifurcating phylogenies and for phylogenies that are incompletely resolved. Several of these methods are available as computer packages, and all are preferable to older methods which did not account for phylogenetic effects. Problems remain, but as the methods we have outlined become further refined, it will be increasingly clear exactly what comparative studies can and cannot tell us across the range of biological enquiry from molecular genetics to community structure. □

Paul H. Harvey and Andy Purvis are in the Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, UK.

1. Darwin, C. *On the Origin of Species by Means of Natural Selection* (Murray, London, 1859).
2. Darwin, C. *The Descent of Man and Selection in Relation to Sex* (Murray, London, 1871).
3. Short, R. V. in *Proceedings of the Canberra Symposium on Reproduction and Evolution*, 3-19 (Australian Academy of Sciences, 1977).
4. Short, R. V. *Adv. Study Behav.* **9**, 131-158 (1979).
5. Short, R. V. in *Reproductive Biology of the Great Apes* (ed. Graham, C. E.) 319-341 (Academic, New York, 1981).
6. Harcourt, A. H., Harvey, P. H., Larson, S. G. & Short, R. V. *Nature* **293**, 55-57 (1981).
7. Harvey, P. H. & Harcourt, A. H. in *Sperm Competition and the Evolution of Animal Mating Systems* (ed. Smith, R. L.) 589-600 (Academic, New York, 1984).
8. Møller, A. P. *J. hum. Evol.* **17**, 479-488 (1988).
9. Møller, A. P. *Funct. Ecol.* **3**, 91-96 (1989).
10. Møller, A. P. *Biol. J. Linn. Soc. Lond.* **33**, 273-283 (1988).
11. Harvey, P. H. & May, R. M. *Nature* **337**, 508-509 (1989).
12. Carpenter, J. M. *Cladistics* **5**, 131-144 (1989).
13. Cavalier Smith, T. *The Evolution of Genome Size* (Wiley, New York, 1985).
14. Burt, A. & Bell, G. *Nature* **326**, 803-805 (1987).
15. Huey, R. B. in *New Directions in Ecological Physiology* (eds Feder, M. E., Bennett, A. F., Burggren, W. & Huey, R. B.) 76-198 (Cambridge University Press, Cambridge, 1987).
16. Trevelyan, R., Harvey, P. H. & Pagel, M. D. *Funct. Ecol.* **4**, 135-141 (1990).
17. Sessions, S. K. & Larson, A. *Evolution* **41**, 1239-1251 (1987).
18. Harvey, P. H. & Pagel, M. D. in *Toward a More Exact Ecology* (eds Grubb, P. J. & Whittaker, J.) 209-230 (Blackwell, Oxford, 1989).
19. Lauder, G. V. *Paleobiology* **7**, 430-442 (1981).
20. Krebs, J. R., Sherry, D. F., Healy, S. D., Perry, V. H. & Vaccarino, A. L. *Proc. natn. Acad. Sci. U.S.A.* **86**, 1388-1392 (1989).
21. Harvey, P. H. & Krebs, J. R. *Science* **249**, 140-146 (1990).
22. Donoghue, M. J. *Evolution* **43**, 1137-1156 (1989).
23. Robertson, J. S. et al. *Virology* **160**, 31-37 (1987).
24. Nee, S. & Maynard Smith, J. M. *Parasitology* **100**, S5-S18 (1990).
25. Margulis, L. *Symbiosis in Cell Evolution* (Freeman, San Francisco, 1981).
26. Harvey, P. H. & Pagel, M. D. *The Comparative Method in Evolutionary Biology* (Oxford University Press, Oxford, 1991).

27. Lack, D. *Ecological Adaptations for Breeding in Birds* (Methuen, London, 1968).
28. Kleiman, D. G. *Q. Rev. Biol.* **52**, 39–69 (1977).
29. Clutton-Brock, T. H. & Harvey, P. H. in *Behavioural Ecology: An Evolutionary Approach* (eds Krebs, J. R. & Davies, N. B.) 7–29 (Blackwell, Oxford, 1984).
30. Ridley, M. *The Explanation of Organic Diversity: The Comparative Method and Adaptations for Mating* (Oxford University Press, Oxford, 1983).
31. Felsenstein, J. *Q. Rev. Biol.* **57**, 379–404 (1982).
32. Harvey, P. H. & Mace, G. M. in *Current Problems in Sociobiology* (King's College Sociobiology Group) 343–361 (Cambridge University Press, Cambridge, 1982).
33. Pagel, M. D. & Harvey, P. H. *Folia Primatol.* **53**, 203–220 (1989).
34. Grafen, A. *Phil. Trans. R. Soc.* **336B**, 119–157 (1989).
35. Grant, P. R. *Ecology and Evolution of Darwin's Finches* (Princeton University Press, Princeton, New Jersey, 1986).
36. Toro, M. & Charlesworth, B. *Heredity* **49**, 199–209 (1982).
37. Clutton-Brock, T. H., Albon, S. D. & Harvey, P. H. *Nature* **285**, 565–567 (1980).
38. Harvey, P. H., Kavanagh, M. & Clutton-Brock, T. H. *Nature* **276**, 817–818 (1978).
39. Packer, C. *Science* **221**, 1191–1193 (1983).
40. Clutton-Brock, T. H. & Harvey, P. H. in *Advances in the Study of Mammalian Behavior* (eds Eisenberg, J. F. & Kleiman, D. G.) 632–663 (American Society of Mammalogists, Shippensburg, Pennsylvania, 1983).
41. Harvey, P. H., Bull, J. J., Pemberton, M. & Paxton, R. J. *Am. Nat.* **119**, 710–719 (1982).
42. Friday, A. *Folia Primatol.* **53**, 221–234 (1989).
43. Clutton-Brock, T. H. & Harvey, P. H. *J. Zool.* **183**, 1–33 (1977).
44. Stearns, S. C. *Oikos* **41**, 173–187 (1983).
45. Cheverud, J. M., Dow, M. M. & Leutenegger, W. *Evolution* **39**, 1335–1351 (1985).
46. Dunham, A. E. & Miles, D. B. *Am. Nat.* **126**, 231–257 (1985).
47. Felsenstein, J. *Am. Nat.* **125**, 1–15 (1985).
48. Martin, R. D. & Harvey, P. H. in *Size and Scaling in Primate Biology* (ed. Jungers, W. L.) 147–173 (Plenum, New York, 1985).
49. Bell, G. *Am. Nat.* **133**, 553–571 (1989).
50. Maddison, W. P. *Evolution* (1990).
51. Gittleman, J. L. & Kot, M. *Syst. Zool.* **39**, 227–241 (1990).
52. Pagel, M. D. & Harvey, P. H. *Q. Rev. Biol.* **63**, 413–440 (1988).
53. Huey, R. B. & Bennett, A. F. in *Predator-Prey Relationships: Perspectives and Approaches for the Study of Lower Vertebrates* (eds Feder, M. E. & Lauder, G. V.) 82–96 (Chicago University Press, Chicago, Illinois, 1986).
54. Huey, R. B. & Bennett, A. F. *Evolution* **41**, 1098–1115 (1987).
55. Losos, J. B. *Anim. Behav.* **39**, 879–890 (1990).
56. Losos, J. B. *Ecol. Monogr.* **60**, 369–388 (1990).
57. Sillén-Tullberg, B. *Evolution* **42**, 293–305 (1988).
58. Ridley, M. *Anim. Behav.* **34**, 1848–1858 (1986).
59. Ridley, M. *Biol. Rev.* **63**, 509–549 (1988).
60. Cope, E. D. *Am. Nat.* **19**, 140–353 (1885).
61. Harvey, P. H. & Paxton, R. J. *Oikos* **37**, 391–393 (1981).
62. Cavalli-Sforza, L. L. & Edwards, A. W. F. *Proc. XI int. Cong. Genet.* **3**, 923–933 (1964).
63. Felsenstein, J. *Syst. Zool.* **22**, 240–249 (1973).
64. Crisci, J. V. & Stuessy, T. F. *Syst. Bot.* **5**, 112–135 (1980).
65. Futuyma, D. J. *Evolutionary Biology* (Sinauer, Sunderland, Massachusetts, 1986).
66. Maddison, W. P., Donoghue, M. J. & Maddison, D. R. *Syst. Zool.* **33**, 83–103 (1984).
67. Maddison, W. P. & Maddison, D. R. *Folia Primatol.* **53**, 190–202 (1989).
68. Höglund, J. *Am. Nat.* **134**, 72–87 (1989).
69. Felsenstein, J. *Ann. Rev. ecol. Syst.* **19**, 445–471 (1988).
70. Burt, A. *Oxf. Surv. evol. Biol.* **6**, 33–53 (1989).
71. Harvey, P. H. *Trends Ecol. Evol.* **6**, 38–39 (1991).
72. Martins, E. P. & Garland, T. H. *Evolution* (1991).
73. Mitter, C., Farrell, B. & Wiegmann, B. *Am. Nat.* **132**, 107–128 (1988).
74. Read, A. F. *Am. Nat.* (in the press).
75. Blackburn, T. M. thesis, Oxford University.
76. Maddison, W. P. *Cladistics* **5**, 365–377 (1989).
77. Rohlf, F. J., Chang, W. S., Sokal, R. R. & Kim, J. *Evolution* **44**, 1671–1684 (1990).
78. Coddington, J. A. *J. Cladistics* **1**, 102–107 (1985).
79. Møller, A. P. & Birkhead, T. R. *Am. Nat.* (in the press).

ACKNOWLEDGEMENTS. We thank J. Felsenstein, A. E. Keymer, A. Ø. Mooers, S. Nee, C. R. Packer, L. Partridge, A. F. Read, J. Reynolds and M. Ridley for comments on the manuscript. We are also grateful to J. Felsenstein, T. Garland, A. Grafen and M. D. Pagel for many helpful discussions about the methods discussed. Our work is funded by the Science and Engineering Research Council and the Commission for the European Communities.

## ARTICLES

# Crystal structure of an N-terminal fragment of the DNA gyrase B protein

Dale B. Wigley, Gideon J. Davies, Eleanor J. Dodson, Anthony Maxwell\* & Guy Dodson

Department of Chemistry, York University, Heslington, York YO1 5DD, UK

\* Department of Biochemistry, University of Leicester, Leicester LE1 7RH, UK

The crystal structure of an N-terminal fragment of the *Escherichia coli* DNA gyrase B protein, complexed with a nonhydrolysable ATP analogue, has been solved at 2.5 Å resolution. It consists of two domains, both containing novel protein folds. The protein fragment forms a dimer, whose N-terminal domains are responsible for ATP binding and hydrolysis. The C-terminal domains form the sides of a 20 Å hole through the protein dimer which may play a role in DNA strand passage during the supercoiling reaction.

DNA gyrase is a type II topoisomerase which catalyses the negative supercoiling of DNA in prokaryotes (for reviews see refs 1, 2). The enzyme from *Escherichia coli* consists of two proteins (A and B) of relative molecular mass 97,000 and 90,000 ( $M_r$  97K and 90K), respectively, with the active protein being an  $A_2B_2$  complex. Gyrase is the target of two groups of antibiotics: the quinolones (for example nalidixic acid and ciprofloxacin) and the coumarins (for example coumermycin  $A_1$  and novobiocin). The quinolones are thought to act at the A protein, whereas the coumarins act at the B protein. Mechanistic studies have revealed the steps that are likely to be involved in the DNA supercoiling process (reviewed in ref. 3). The enzyme binds to

DNA and forms a complex in which about 120 base pairs (bp) of DNA are wrapped around a protein core. The wrapped DNA is then cleaved in both strands with the concomitant formation of covalent bonds between the protein and the DNA. A segment of DNA is passed through this break, and probably through part of the protein itself. Supercoiling of DNA by gyrase requires the hydrolysis of ATP but, in the absence of nucleotide, gyrase will relax negatively supercoiled DNA. Replacement of ATP by the nonhydrolysable ATP analogue ADPNP (5'-adenylyl- $\beta$ - $\gamma$ -imidodiphosphate) results in limited supercoiling by gyrase, suggesting that ATP binding can promote a single round of supercoiling but that hydrolysis is required to regenerate the enzyme in an active form<sup>4</sup>.

Low-resolution structural studies<sup>5–7</sup> have shown that the gyrase-DNA complex resembles a flattened sphere and may be 'heart-shaped', with the bound DNA partially embedded in the protein. There is evidence for channels or cavities in the complex which may have a role in the DNA translocation process. The detailed structure is not known and no crystals of X-ray diffraction quality of either of the gyrase A or B proteins have been reported.

The A protein can be cleaved by trypsin into two fragments: a 64K N-terminal fragment which contains the DNA breakage-reunion site, and a C-terminal 33K fragment which seems to be stabilize the DNA-protein complex<sup>8,10</sup>. Genetic constructs have been produced which overexpress these fragments individually<sup>9,10</sup>, and the 64K fragment has been crystallized<sup>11</sup>.

Some bacterial strains produce a 47K protein which comprises



TABLE 1 Data collection and phasing statistics

## DATA COLLECTION

| Data                             | Method of collection | Resolution (Å) | $R_{\text{sym}}$ (%) | No. of measurements | Unique data (% completeness) |
|----------------------------------|----------------------|----------------|----------------------|---------------------|------------------------------|
| Native                           | film                 | 2.5            | 7.9                  | 40,769              | 15,725 (86.3)                |
| Thimerosal                       | film                 | 2.5            | 5.7                  | 42,395              | 13,596 (75.3)                |
| K <sub>2</sub> PtCl <sub>4</sub> | diffractometer       | 6.0            | —                    | 1,296               | 1,296 (92.0)                 |
| NaAuCl <sub>4</sub>              | diffractometer       | 6.0            | —                    | 1,313               | 1,313 (93.2)                 |

## PHASING STATISTICS

| Derivative                       | Anomalous data | No. of sites | $R_{\text{deriv}}$ (%) | $R_{\text{cutis}}$ (%) | Phasing power |
|----------------------------------|----------------|--------------|------------------------|------------------------|---------------|
| Thimerosal                       | yes            | 2            | 22.0                   | 40.6                   | 3.24          |
| K <sub>2</sub> PtCl <sub>4</sub> | no             | 2            | 26.9                   | 64.7                   | 1.39          |
| NaAuCl <sub>4</sub>              | no             | 1            | 20.1                   | 84.5                   | 1.20          |

Diffractometer data were collected using a four-circle diffractometer coupled to a rotating anode source. Only the unique data were collected. Three reflections were chosen as standards to monitor crystal decay, and were measured after every 100 reflections had been recorded. The data were also corrected for absorption. Film data were collected on 2 or 3° oscillation photographs, at the LURE synchrotron source, using 1.47 Å wavelength radiation. Care was taken to align the thimerosal derivative crystals such that anomalous pairs were recorded on the same film, whereas native crystals were deliberately misset by 10° to reduce loss of data in the 'blind region'. Films were digitized using a Joyce-Loebl Scandig-3 film scanner, with a 50 µm raster step size, and processed using MOSFLM (Imperial College, London) and the CCP4 programs (Daresbury, UK). A 2.5 Å resolution electron density map was calculated, using MIR phases up to 6.0 Å resolution and SIR phases between 6.0 and 2.5 Å. The resulting map was solvent flattened<sup>26</sup>, and model built from inspection of the maps before and after solvent flattening. The first model comprised some 65% of the residues, including side chains. This partial model was then used in phase combination<sup>17</sup> to improve the poorer regions of the map. The next round of model building allowed inclusion in the model of 90% of the residues. A further round of phase combination was applied, and a map calculated which allowed all but 12 residues to be included in the model. These residues (303–314) form a flexible loop. After several cycles of Hendrickson–Konnert refinement<sup>18</sup> the *R*-factor had fallen to 28%. The model was then improved by a round of molecular dynamics refinement<sup>19</sup> after which the *R*-factor had fallen to 24% and was accompanied by a considerable improvement in the overall geometry of the model. At this stage the bound ADPNP and solvent molecules were included in the model, and the refinement was completed by a few cycles of Hendrickson–Konnert refinement. The present model contains 391 residues, one ADPNP molecule, one magnesium ion, and 83 solvent molecules. The current *R*-factor for all data between 10.0 and 2.5 Å resolution is 17.8%, with r.m.s. deviation from ideality in bond lengths and planarity of 0.020 Å and 0.019 Å, respectively.

the C-terminal half of the B protein<sup>12–14</sup>. When complexed with the A protein, this fragment will support relaxation but not supercoiling of DNA. Consequently, the N-terminal region of the B protein probably contains the site of ATP hydrolysis<sup>12,14</sup>. A genetic construct has been produced that overexpresses a 43K N-terminal fragment of the B protein comprising residues 2–393 of the intact protein. This protein binds novobiocin and hydrolyses ATP (A. P. Jackson and A.M., unpublished data) and has been crystallized in the presence of ADPNP<sup>15</sup>. We report here the crystal structure of this complex at 2.5 Å resolution.

### Structure determination

Crystals were prepared as described previously<sup>15</sup>. The crystals used for the structure determination were of the orthorhombic space-group C222<sub>1</sub> with cell dimensions  $a = 89.2$  Å,  $b = 143.1$  Å and  $c = 79.8$  Å, containing one molecule in the asymmetric unit. Three heavy-atom derivatives were identified (Table 1), but of these, high resolution data from the mercury derivative alone was sufficient to solve the structure. Consequently, the other derivatives were not analysed beyond low resolution.

The initial solvent-flattened map, calculated at 2.5 Å resolution, was readily interpretable, with the aid of the known amino-acid sequence<sup>14,16</sup>, and allowed a complete and unambiguous assignment of a domain at the C terminus, and its connection to a second domain. The first 50 residues at the N terminus could also be identified. The density corresponding to the rest of the N-terminal domain, although still quite good, was improved further by phase combination<sup>17</sup> using phases

calculated from the partial model. Initial rounds of crystallographic refinement used the restrained least squares minimization method of Hendrickson and Konnert<sup>18</sup>. When the chain connectivity was correct, refinement was continued using molecular dynamics<sup>19</sup>, before completion by a few cycles of model building followed by Hendrickson–Konnert refinement. The final rounds of model building were used to insert solvent molecules and the ADPNP molecule. The current *R*-factor of our model is 17.8%. The coordinates will be deposited at the Brookhaven Databank. Details of the structure determination and a fuller description of the structure will be presented elsewhere.

### Structure of the monomer

The 43K fragment contains two distinct crystallographic domain which we will refer to as domain 1 and domain 2 (Fig. 1). Domain 1 (residues 2–220; the N-terminal methionine residue is removed *in vivo*) contains the ATP-binding site. The fold of this domain (Fig. 1Ca) is unique among known protein structures. It comprises an eight-stranded  $\beta$  sheet which can be subdivided into a six-stranded and a two-stranded antiparallel sheet connected by a parallel-stranded section. The strand connectivity is also rather unusual. Interestingly, there are five helical regions, one of which contributes to the ATP-binding site (see below). The structure of the domain is stabilized by a hydrophobic core.

The fold of domain 2 (residues 221–392) is also unique (Fig. 1Cb). The central core of the domain consists of a four-stranded  $\beta$  sheet, but again the connectivity is unusual. The sheet is neither parallel nor antiparallel, but mixed. This sort of sheet has also been found in a number of other proteins (for example endothiapepsin<sup>20</sup>, carboxypeptidase<sup>21</sup>, pancreatic lipase<sup>22</sup>), but the rest of the domain is not similar to any of these proteins. In particular, at the C terminus there are two long helices, each containing about twenty-five residues. The second of these helices (residues 366–392) protrudes from the main body of the protein. Of the last 14 residues of this helix, six are arginines. The implications of which are discussed below. The remainder of the domain comprises two further helices. The first lies at an angle of around 30° to the first of the two C-terminal helices and forms a large part of the surface of this domain. This helix also carries a number of arginine residues. A fourth helix lies across the other side of the sheet. As with domain 1 there is a large hydrophobic core to the domain.

The whole structure is tightly folded with short connecting regions between the secondary structural elements. Proline residues are important for the tight conformation of the fragment. For example, at four positions in the structure there are two proline residues within five residues of each other, and in all of these places there is a sharp turn in the protein fold.

### Dimer interactions

Biochemical evidence indicates that the 43K fragment is a dimer in the presence of ADPNP (see below). With one molecule in the asymmetric unit in this space-group there are two possible pairs of molecules which could make up a physiological dimer. For one of these two alternatives, the molecular contacts are much more extensive, so these two molecules are presumed to form the dimer. Most of the contacts between the molecules of the dimer are through the N-terminal domains (Fig. 3), the most important being through the N-terminal arm (residues 2–15), which protrudes from the surface of the monomer and wraps around domain 1 of the other subunit. This interaction gives rise to several important contacts between the two molecules which stabilize the dimer interface and even form part of the ATP-binding site (see below). There are relatively few other contacts between the two N-terminal domains, these being unusually hydrophilic for a dimer interface, suggesting that the interaction is rather weak.

At the C terminus of the protein, there are contacts between

the C-terminal helices of the two monomers, despite both carrying a large net positive charge. But given that this structure is of a protein which has been truncated at the C terminus, it is prudent to be cautious about interpreting the structure in this region which may not be preserved in the full-length protein.

### ATP-binding site

Electron density corresponding to the bound ADPNP was found at the centre of domain 1. Binding of the ATP analogue appears to be stabilized by several interactions with the protein (Fig. 4). One important residue is Lys at position 103 (Lys 103) which has been identified by affinity-labelling studies as possibly contributing to the ATP-binding site<sup>23</sup>. This residue forms a salt-bridge with the  $\beta$ -phosphate of the ADPNP. The bound  $Mg^{2+}$  ion can also be identified, along with two coordinated water molecules. The six coordination ligands of the  $Mg^{2+}$  comprise these two waters and oxygens from each of the three phosphates, with the final coordination site being occupied by the oxygen from the amide side chain of Asn 46. Interestingly, there are interactions between the side chains of Gln 335 and Lys 337 with the  $\gamma$ -phosphate of the ADPNP. These residues are not in domain 1 but reach up from domain 2. Consequently the contacts between ATP and domain 2 may provide a mechanism for signalling the hydrolysis of ATP to the molecule as a whole. This may be of importance for defining the relative positions of the two domains. The presence of two consecutive glycine

residues (220 and 221) at the link between domains 1 and 2 does suggest that this region is flexible.

The adenine ring makes a number of polar contacts with the protein, though in general the sides of the adenine-binding pocket are fairly hydrophobic. In particular, Tyr 109 forms a hydrogen bond with the N3 atom of the adenine ring, and the amino side-group of the ring interacts with the side chain of Asp 73. This latter interaction may explain the specificity for ATP, over GTP, as cofactor for supercoiling<sup>4</sup>.

Many ATP-binding sites contain a common structural motif with the phosphates of the nucleotide being bound at one end of a helix adjacent to a number of glycine residues, which allow the phosphates to tuck in close to the end of the helix<sup>24</sup>. It is thought that this binding motif allows maximization of the binding energy which can be utilized from the helix dipole. Such a binding motif is also found in this structure with residues 118–126 forming a short helix after a glycine-rich region. The phosphates of the ADPNP lie against glycine residues 114, 117 and 119. A role for these glycines in ATP binding has been suggested previously<sup>23</sup>.

A most intriguing aspect of the ATP-binding site is the interaction between the bound ADPNP with Tyr 5 on the N-terminal arm of the other molecule of the dimer, thus both molecules contribute to the nucleotide-binding site. This interaction is with the 2' hydroxyl of the ribose ring and probably explains why 2'-deoxy-ATP will not support supercoiling by gyrase<sup>4</sup>.

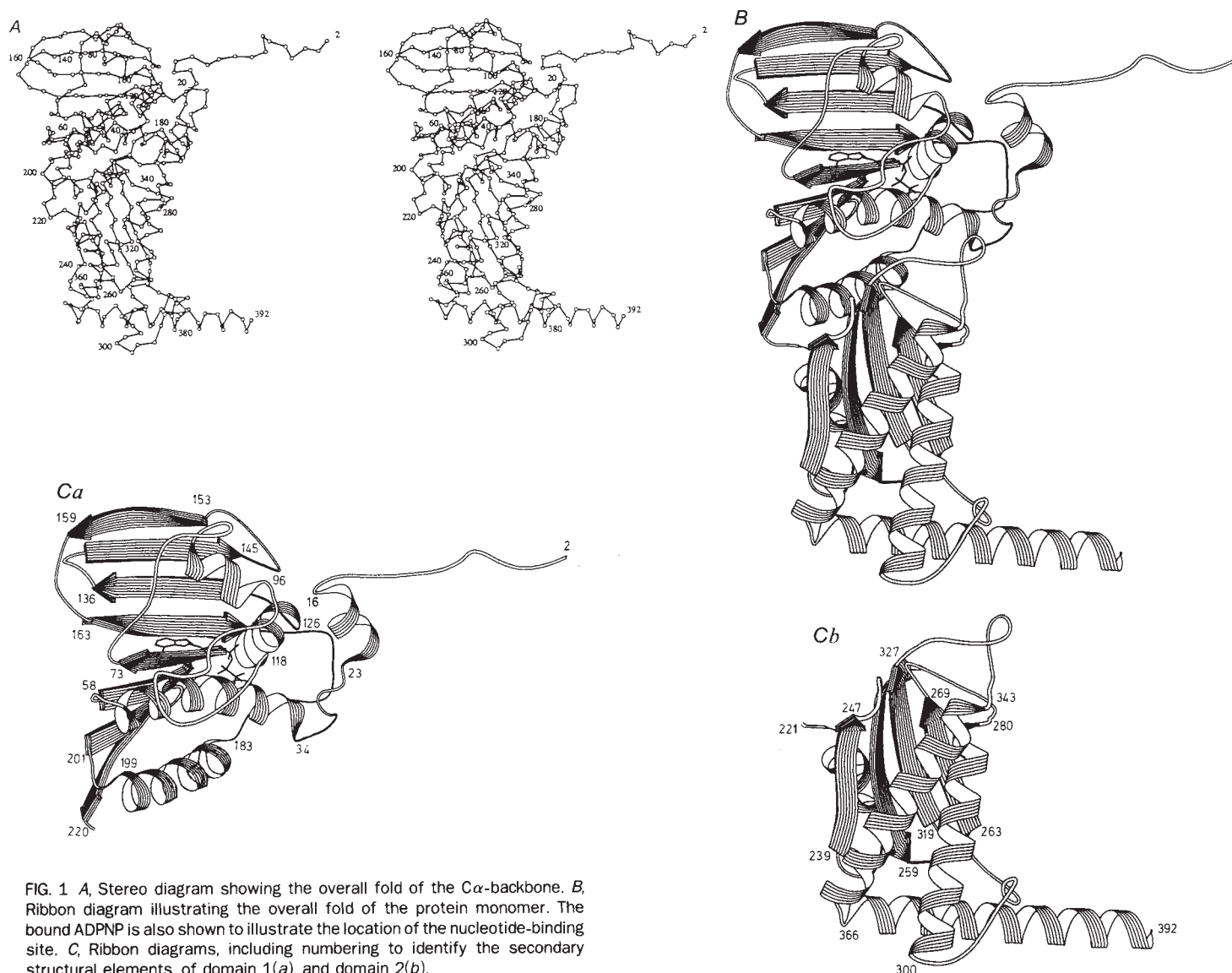


FIG. 1 A, Stereo diagram showing the overall fold of the  $\alpha$ -backbone. B, Ribbon diagram illustrating the overall fold of the protein monomer. The bound ADPNP is also shown to illustrate the location of the nucleotide-binding site. C, Ribbon diagrams, including numbering to identify the secondary structural elements, of domain 1(a), and domain 2(b).



The ATPase activity of the 43K fragment shows a greater than first-order dependence upon protein concentration (A. P. Jackson, J. A. Ali and A.M., unpublished data). This observation could be explained if binding of ATP were to induce the formation of dimers, or higher oligomers, of this fragment.  $M_r$  estimations from sedimentation equilibrium experiments, indicate that the 43K protein does indeed form dimers in the presence of ADPNP, but is a monomer in the absence of this analogue (A. J. Rowe and A.M., unpublished data). The interactions between the ADPNP and the protein in the crystal structure also suggest that a protein dimer is required to bind ATP. These observations have important implications for the mechanism of supercoiling by gyrase.

### Mechanistic implications

Existing biochemical data suggest that the A protein of gyrase

is principally involved in the interaction with DNA, whereas the B protein is the site of ATP hydrolysis. Current models for the mechanism of supercoiling by gyrase<sup>3</sup> involve the passage of DNA through a double-stranded break, which is held open by the protein. Such models require passage of the DNA through part of the protein as well. Several cycles of supercoiling can be catalysed by gyrase without dissociation of the protein from the DNA<sup>25</sup> (known as 'processive' supercoiling). Consequently, if DNA is to pass through part of the protein molecule during supercoiling, then this passage is likely to be through some kind of gateway rather than by dissociation of two or more parts of the protein. It is evident from Fig. 3 that there is a large hole running through the protein dimer, which has a diameter of around 20 Å. Given that the diameter of a DNA helix is also about 20 Å, it is possible that this hole may form a part of just such a gateway through which the DNA is passed during super-

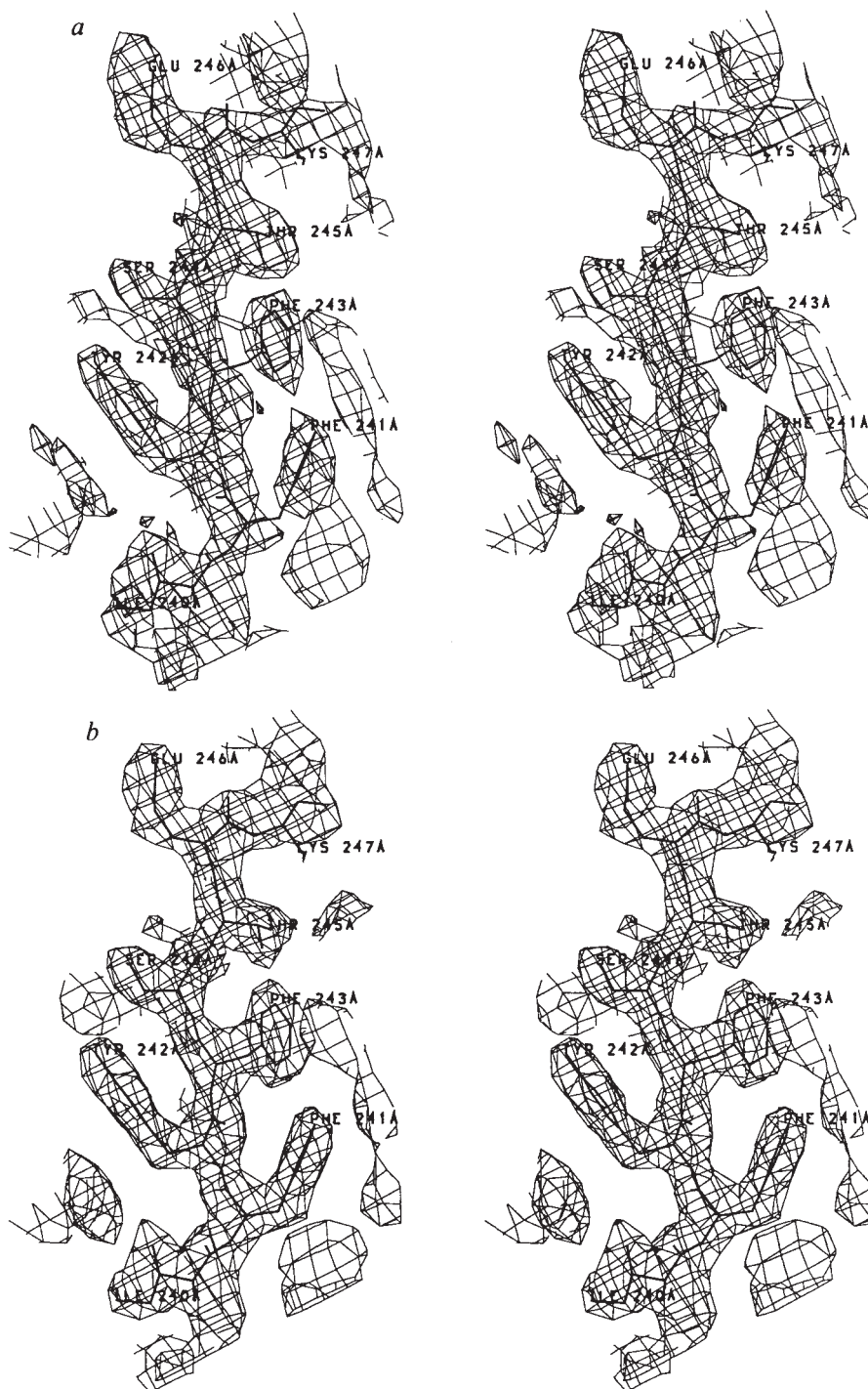


FIG. 2 Representative sections of electron density, contoured at a level of  $1.0\sigma$  in both cases and corresponding to residues 240–247 in *a*, the initial MIR map, before solvent-flattening, and *b*, the final  $2F_o - F_c$  map. The coordinates of the final model are superimposed for reference.

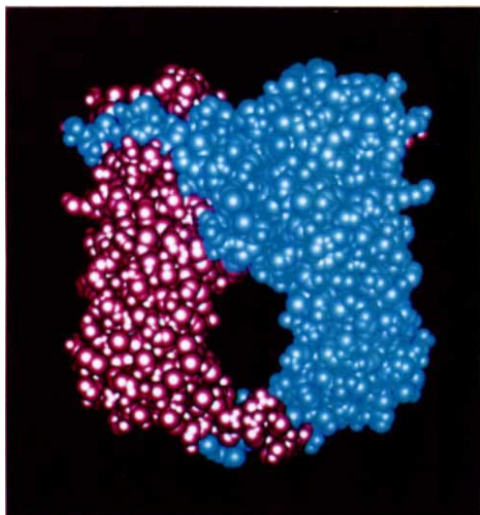
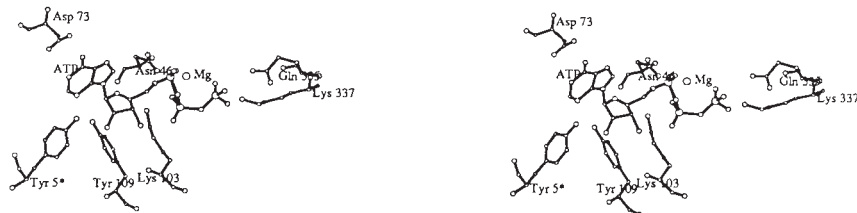


FIG. 3 Space-filling model of the protein dimer, with each subunit picked out in a different colour, illustrating the contacts between subunits.

coiling. It is also interesting that in domain 2, every arginine residue protrudes into this remarkable cavity (Fig. 5). The unusually arginine-rich C-terminal helix contributes to this site, by forming the lower rim which closes off the hole in this structure. It is conceivable that these arginine residues form a DNA-binding surface within the gateway.

It has been suggested<sup>4</sup> that the role of ATP in the supercoiling reaction is to regenerate gyrase in an active form. If ADPNP is substituted for ATP in the supercoiling reaction, limited supercoiling is observed which is thought to correspond to a single

FIG. 4 Stereo diagram of residues which form the ATP-binding site. Note that Tyr 5 is part of a different polypeptide chain to the other residues.



turnover by gyrase<sup>4</sup>. This result implies that the DNA strand-passage event has occurred before hydrolysis of ATP and that, because ADPNP cannot be hydrolysed by gyrase, the enzyme is trapped in a form which is unable to catalyse further rounds of supercoiling. The crystal structure presented here suggests a possible explanation, with implications for the role of ATP hydrolysis in the supercoiling reaction. We propose that in the first step of the reaction, gyrase binds to DNA. The second step involves cleavage of the DNA by the A proteins. The next step has to involve a large conformational change in the protein to allow passage of another section of DNA, through the double-stranded break, into the interior of the protein complex. It is this step which may involve binding of ATP which would stabilize the interactions between the domains at the N termini of the B proteins. This conformational change could be transmitted to the A proteins allowing DNA to pass into the interior of the protein complex. The double-stranded break could then be religated, leaving two supercoils in the DNA. During processive supercoiling, however, it is possible that the DNA need not be religated between successive cycles of supercoiling, and that religation may simply be a requirement for dissociation of the protein-DNA complex. The final step in the supercoiling cycle involves release of the DNA from the interior of the complex. We suggest that it is this step which requires hydrolysis of ATP to weaken the interaction between the N-terminal domains of the B protein, allowing them to move apart and open a gap through which the DNA can be released.

Thus we propose that ATP hydrolysis is coupled to supercoiling in the following way. The binding energy for the association between ATP and the B protein is used to stabilize an otherwise unfavourable conformation of the protein. This energy is released when ATP is hydrolysed to ADP and inorganic phosphate, which dissociate from the protein allowing it to 'relax' back to the conformation at the start of the cycle. It is

FIG. 5 The distribution of arginine residues in the vicinity of the 20 Å hole through the protein dimer. All of the arginine residues in domain 2 (residues 220–392) are shown in blue. For reference, space-filling representations of the bound ADPNP molecules are also shown.



because this last step is not possible with ADPNP that only a single round of supercoiling is supported. Although electric dichroism and neutron scattering studies<sup>6,7</sup> indicate that there is indeed a conformational change on addition of ADPNP,

which would support this hypothesis, it is clear that X-ray structural analysis of larger assemblies and complexes of the gyrase system is needed to provide further information about the mechanism of supercoiling by gyrase. □

Received 25 February; accepted 13 May 1991.

- Gellert, M. A. *Rev. Biochem.* **50**, 879–910 (1981).
- Wang, J. C. A. *Rev. Biochem.* **54**, 665–697 (1985).
- Maxwell, A. & Gellert, M. *Adv. Protein Chem.* **38**, 69–107 (1986).
- Sugino, A., Higgins, N. P., Brown, P. O., Peebles, C. L. & Cozzarelli, N. R. *Proc. natn. Acad. Sci. U.S.A.* **75**, 4838–4842 (1978).
- Kirchhausen, T., Wang, J. C. & Harrison, S. C. *Cell* **41**, 933–943 (1985).
- Rau, D. C., Gellert, M., Thoma, F. & Maxwell, A. *J. molec. Biol.* **193**, 555–569 (1987).
- Krueger, S. *et al. J. molec. Biol.* **211**, 211–220 (1990).
- Reece, R. J. & Maxwell, A. *J. biol. Chem.* **264**, 19648–19653 (1989).
- Reece, R. J. & Maxwell, A. *J. biol. Chem.* **266**, 3540–3546 (1991).
- Reece, R. J. & Maxwell, A. *Nucleic Acids Res.* **19**, 1399–1405 (1991).
- Reece, R. J., Dauter, Z., Wilson, K. S., Maxwell, A. & Wigley, D. B. *J. molec. Biol.* **215**, 493–495 (1990).
- Brown, P. O., Peebles, C. L. & Cozzarelli, N. R. *Proc. natn. Acad. Sci. U.S.A.* **76**, 6110–6114 (1979).
- Gellert, M., Fisher, L. M. & O'Dea, M. *Proc. natn. Acad. Sci. U.S.A.* **76**, 6289–6293 (1979).
- Adachi, T. *et al. Nucleic Acids Res.* **15**, 771–784 (1987).
- Jackson, A. P., Maxwell, A. & Wigley, D. B. *J. molec. Biol.* **217**, 15–17 (1991).
- Yamagishi, J.-I., Yoshida, H., Yamayoshi, M. & Nakamura, S. *Molec. Gen. Genet.* **204**, 367–373 (1986).

- Rice, D. W. *Acta Crystallogr.* **A37**, 491–500 (1981).
- Hendrickson, W. A. & Konnert, J. H. in *Structure, Conformation and Evolution* vol. 1 (ed. Srinivasan, R.) 43–57 (Pergamon, Oxford, 1981).
- Brünger, A. T. *J. molec. Biol.* **203**, 803–816 (1988).
- Subramanian, E. *et al. Proc. natn. Acad. Sci. U.S.A.* **74**, 556–559 (1977).
- Rees, D. C., Lewis, M. & Lipscomb, W. N. *J. molec. Biol.* **168**, 367–387 (1983).
- Winkler, F. K., D'Arcy, A. & Hunziker, W. *Nature* **343**, 771–774 (1990).
- Tamura, J. K. & Gellert, M. *J. biol. Chem.* **265**, 21342–21349 (1990).
- Wierenga, R. K., De Maeyer, M. C. H. & Hol, W. G. J. *Biochemistry* **24**, 1346–1357 (1985).
- Morrison, A., Higgins, N. P. & Cozzarelli, N. R. *J. biol. Chem.* **255**, 2211–2219 (1980).
- Wang, B. C. *Meth. Enzym.* **115**, 90–112 (1985).

ACKNOWLEDGEMENTS. We thank R. Fourme and his staff for generously providing access to the data collection facilities at the LURE synchrotron at extremely short notice, A. P. Jackson for protein and unpublished data, R. E. Hubbard and M. J. Hartshorn for assistance with the preparation of molecular graphics figures, H. F. J. Savage for advice, S. E. Halford and A. J. Wilkinson for critical reading of the manuscript and J. A. Ali, A. J. Grimshaw, K. S. Lilley, R. J. Reece and A. J. Rowe for their contributions to the work. The project was jointly supported by the Wellcome Trust and SERC. D.B.W. is an SERC Advanced Fellow, and A.M. is a Lister Institute Jenner Fellow.

## LETTERS TO NATURE

### Soft X-ray shadowing by the Draco cloud

D. N. Burrows & J. A. Mendenhall

Department of Astronomy and Astrophysics,  
The Pennsylvania State University,  
525 Davey Lab, University Park, Pennsylvania 16802, USA

THE astronomical soft X-ray background has both extragalactic and, at lower energies, galactic components. Using the Roentgen Astronomy Satellite (ROSAT), we have detected shadowing of the X-ray background in the  $\frac{1}{4}$  keV band (presumed to be of galactic origin) by the unusual interstellar cloud in Draco. The Draco cloud is at high galactic latitude and, at several hundred parsecs distance from the Sun, is well away from the galactic plane. It reduces the  $\frac{1}{4}$  keV emission by 63% relative to the adjacent sky, implying that a substantial contribution to the X-ray background in this direction comes from hot gas beyond the cloud. This is the first direct evidence for a million-degree galactic halo of gas, but the significance of this observation for understanding the global diffuse X-ray background is unclear, as it contradicts earlier studies<sup>1–3</sup> which failed to find shadowing by galactic gas. More observations of this sort, covering objects distributed throughout the galaxy, are needed to explore the morphology of the hot and cold gas in the nearby interstellar medium.

According to spatial and spectral evidence, the soft X-ray background (SXRb) consists of three components. From 2 to 15 keV, the X-ray background is dominated by an isotropic, extragalactic flux with a power-law spectral form. Two diffuse galactic components dominate in the  $\frac{1}{4}$  keV and  $\frac{3}{4}$  keV ranges. In the  $\frac{1}{4}$  keV band the emission is highly anisotropic, anticorrelated with the column density of neutral gas ( $N_{\text{H}}$ ), and consistent with the spectrum produced by a plasma with a temperature of  $\sim 10^6$  K and no absorption. The  $\frac{3}{4}$  keV emission is nearly isotropic and requires an additional spectral component.

The structure of the soft X-ray emission along the line of sight can be determined by observations of X-ray shadows cast by absorbing clouds at known distances. Such observations can place limits on the distance to the emitting gas and can be compared with models of the SXRb<sup>4–6</sup>. Shadowing studies performed over the past two decades<sup>1–3,7</sup> have placed upper limits of 10–20% on the fraction of the  $\frac{1}{4}$  keV flux at the Earth that originates beyond the neutral gas of the galactic disk. As a result of these observations and other evidence, the  $\frac{1}{4}$  keV diffuse background is generally believed to be dominated by emission

from a region of hot, low-density plasma surrounding the Sun (the 'Local Bubble')<sup>6</sup>. But instrumental limitations have restricted these studies to a handful of directions, and the three-dimensional distribution of the emitting gas is still poorly known.

The ROSAT position-sensitive proportional counter<sup>8</sup> is an ideal instrument for performing shadowing observations towards high-latitude clouds because of the large effective area and fast optics of the telescope, and the large field of view and low internal background rate of the imaging detector. Here we present preliminary results from ROSAT observations of a prominent interstellar cloud in the constellation Draco. Similar results have recently been obtained from the ROSAT sky survey for a different portion of the Draco cloud with about a quarter of the exposure time of our pointed observation<sup>9</sup>. These observations provide the first direct evidence for a more complex structure of the SXRb than is implied by the Local Bubble model<sup>6</sup>.

The Draco cloud<sup>10</sup> is located at galactic longitude and latitude ( $l, b$ )  $\approx (90^\circ, +39^\circ)$ . It has a distinctive narrow 21-cm line centred at a LSR (local standard of rest) velocity of  $\sim -21$  km s<sup>-1</sup> with a width of  $\sim 2$  km s<sup>-1</sup>, which facilitates its separation from the foreground or background emission. Its unusual velocity and 'comet-like' morphology suggest a hydrodynamic interaction with the interstellar medium<sup>11</sup>. The distance of this cloud is uncertain by at least a factor of two, but the most reliable data<sup>12</sup> provide a lower limit of 300 pc, which places it at least 200 pc above the plane of the galaxy. At this distance, it should not absorb X-ray emission from the interior of the Local Bubble, which is believed to extend to  $\sim 150$ –200 pc in this direction<sup>6</sup>.

Our soft X-ray observations were performed as part of the on-orbit verification of the ROSAT instruments and scheduling software on 23–24 July 1990. Figure 1 shows smoothed images of the diffuse X-ray emission obtained from a 4,158-s observation. A 100- $\mu$ m IRAS (infrared astronomy satellite) map of the Draco cloud is superimposed on each X-ray image.

The detailed anticorrelation between the C-band (0.13–0.28 keV, or roughly  $\frac{1}{4}$  keV) intensity and the 100- $\mu$ m intensity in Fig. 1a is striking. There is little doubt that the X-ray feature is produced by absorption, and not by some chance coincidence of X-ray and 100- $\mu$ m features, by displacement or by emission associated with the edge of the cloud (note the faint X-ray filament at right ascension 16h 46.8 min declination  $+60^\circ 4'$  and the X-ray peaks associated with 100- $\mu$ m minima at (16h 42.7 min,  $+60^\circ 20'$ ) and at (16h 45.7 min,  $+60^\circ 33'$ )). The average C-band intensity towards the cloud is  $(4.9 \pm 0.7) \times 10^{-4}$  counts s<sup>-1</sup> arcmin<sup>-2</sup>, the intensity off the cloud varies from  $(9.0 \pm 0.9) \times 10^{-4}$  counts s<sup>-1</sup> arcmin<sup>-2</sup> in the northeastern portion to  $(13.3 \pm 1.4) \times 10^{-4}$  counts s<sup>-1</sup> arcmin<sup>-2</sup> in the southwestern

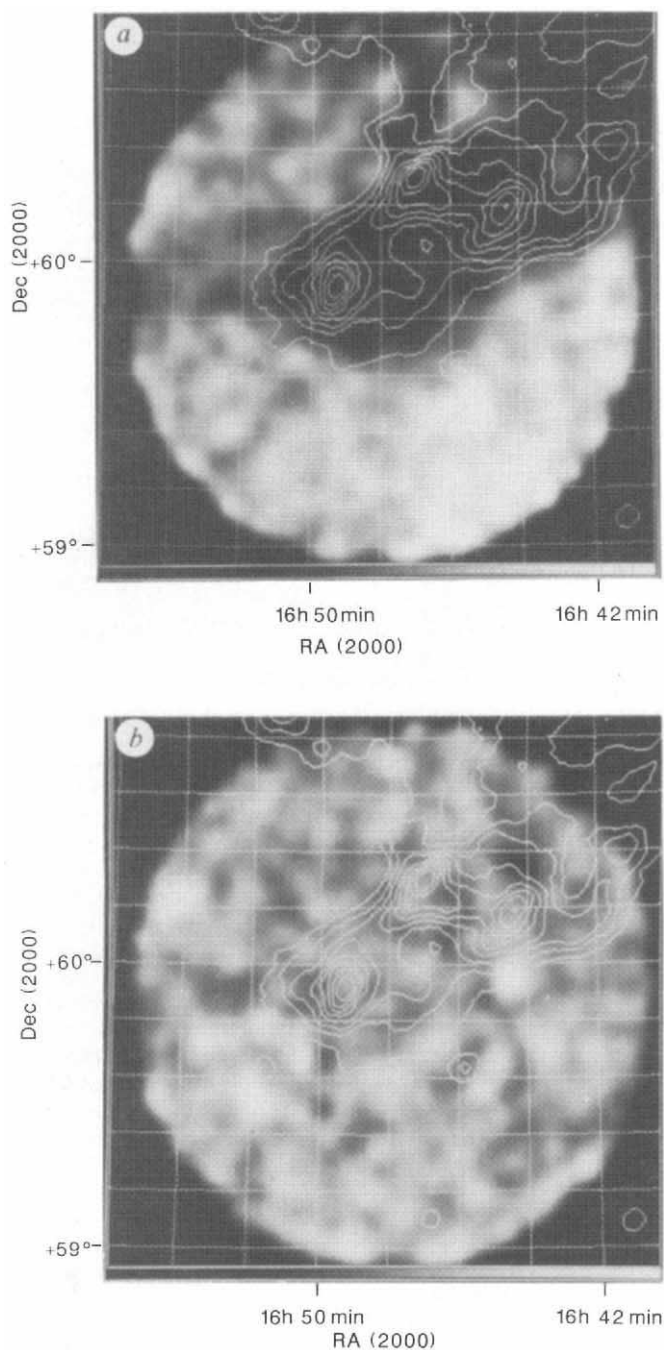


FIG. 1 Images by the ROSAT position-sensitive proportional counter of the Draco cloud in celestial coordinates, smoothed to 5' full width at half maximum (FWHM). The IRAS 100- $\mu\text{m}$  map overlaid on the X-ray image (contour interval of 1 MJy  $\text{sr}^{-1}$ ) was produced from recalibrated summed scan data after correction for baseline variations and subtraction of the zodiacal light. The raw X-ray image was produced by the standard ROSAT processing software. Further processing and analysis were performed using the IRAF/PROS image processing and analysis package and our own software. The raw image was corrected for telescope vignetting, obscuration by the support structure for the detector window and aspect jitter. Point sources were removed manually. The flattened image was then smoothed with a 5' (FWHM) gaussian function. Some residual features are due to imperfect removal of point sources and imperfect flat-fielding. *a*, C-band (0.13–0.28 keV) image. The gradient in C-band intensity across the field may be caused by foreground absorption of the distant X-ray flux. The small-scale structure is larger than expected from counting statistics and may represent real variations in the diffuse emission or absorption at the 10% level. *b*, Hard X-ray image (0.5–2.0 keV). No shadow is apparent at these energies. Most of the small-scale structure in this image is consistent with counting statistics.

portion. (By contrast, the charged particle rate registered by the position-sensitive proportional counter is typically  $10^{-5}$  counts  $\text{s}^{-1}$  arcmin $^{-2}$ . Scattered solar X-rays and other sources of extraneous background are also expected to contribute a small fraction of the sky rate. These background components will tend to decrease the depth of the X-ray shadow.)

There is no apparent absorption in the hard X-ray image of Fig. 1*b*. The hard-band count rates for the same regions used above are  $(2.9 \pm 0.4)$ ,  $(2.9 \pm 0.5)$  and  $(3.5 \pm 0.5) \times 10^{-4}$  counts  $\text{s}^{-1}$  arcmin $^{-2}$ , respectively. Although most of the Draco cloud is transparent to  $\frac{3}{4}$  keV X-rays, with peak H I column densities<sup>10</sup> of  $\sim 1 \times 10^{20} \text{ cm}^{-2}$ , there are three molecular concentrations in our field of view with much higher total column densities, at (16h 49.3 min, +59° 55'), (16h 44.4 min, +60° 12') and (16h 47 min, +60° 18')<sup>13,14</sup>. The 100- $\mu\text{m}$  peaks of 7–8 MJy  $\text{sr}^{-1}$  correspond to total column densities ( $N_{\text{H I}} + 2N_{\text{H}_2}$ ) of  $\sim 9 \times 10^{20} \text{ cm}^{-2}$  assuming a typical gas-to-dust ratio<sup>15</sup>. This represents  $\sim 0.5$  optical depths to the X-rays in our hard image (for the best-fit spectral model), and the cloud should cast a shadow if a substantial fraction of the hard X-rays come from beyond it. The lack of any apparent absorption suggests that the  $\frac{3}{4}$  keV galactic component originates on the near side of the Draco cloud, but a more thorough analysis with an optimally defined  $\frac{3}{4}$  keV band will be necessary before firm conclusions can be drawn.

The fraction of the observed  $\frac{1}{4}$  keV photons emitted beyond the cloud can be determined if we assume that the foreground and background emission are uniform across the field. We have fitted the data with a simple two-component slab absorption model of the form

$$I(i, j) = I_L + I_D \exp(-\langle \sigma_{100} \rangle I_{100}(i, j)) \quad (1)$$

where  $I(i, j)$  is the observed broad-band count rate in the  $(i, j)$ th pixel,  $I_L$  represents the local count rate,  $I_D$  represents the distant count rate,  $\langle \sigma_{100} \rangle$  is the band-averaged X-ray absorption cross-section (measured with respect to  $I_{100}$  in units of (MJy per sr) $^{-1}$ ), and  $I_{100}(i, j)$  is the 100- $\mu\text{m}$  intensity in the  $(i, j)$ th pixel, which is proportional to the total column density.

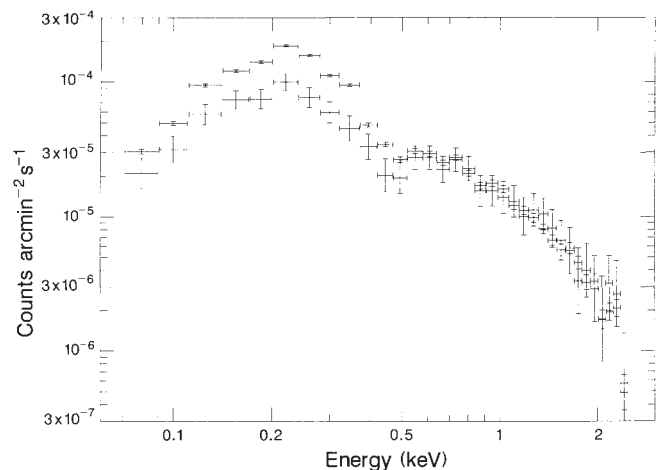
Figure 2 shows fits of this model to our data, and the results are given in Table 1. The best-fit cross-section and the local rate are the same in the two regions, with a variation in  $I_D$  accounting for the intensity variation across the field (this variation may actually be produced by foreground absorption of the distant component by the low-velocity H I, which has a gradient across this field<sup>12</sup>). The best-fit X-ray absorption cross-section,  $\langle \sigma_{100} \rangle$ , corresponds to an effective hydrogen absorption cross-section of  $\sim (0.90 \pm 0.15) \times 10^{-20} \text{ cm}^2$  per hydrogen atom for a typical  $I_{100}/N_{\text{H}}$  ratio<sup>15</sup>, compared with the expected value of  $\sim 0.75 \times 10^{-20} \text{ cm}^2$ . The effective X-ray absorption cross-section derived from these data does not require the small-scale clumping of the neutral material which has been invoked to explain the much smaller effective absorption cross-sections found for global fits of this two-component model to the  $\frac{1}{4}$  keV diffuse background<sup>16</sup>, but which has not been observed<sup>17,18</sup>. Alternatively, if we assume that there is no clumping, the ratio of the standard X-ray absorption cross-section to the best-fit value of  $\langle \sigma_{100} \rangle$  gives a 100  $\mu\text{m}$  to gas ratio of  $I_{100}/N_{\text{H}} = (0.71 \pm 0.11) \times 10^{-20}$  (MJy  $\text{sr}^{-1}$ )/ $\text{cm}^2$ . Under these assumptions the 100- $\mu\text{m}$  data, which predict absorbing column densities for this cloud roughly two to three times as large as the observed  $N_{\text{H I}}$ , are consistent

TABLE 1 C-band absorption model parameters

| Region     | $I_L$<br>(counts $\text{s}^{-1}$<br>arcmin $^{-2}$ ) | $I_D$<br>(counts $\text{s}^{-1}$<br>arcmin $^{-2}$ ) | $\langle \sigma_{100} \rangle$<br>(MJy $^{-1}$ sr) | $\chi^2$ | Frequency |
|------------|------------------------------------------------------|------------------------------------------------------|----------------------------------------------------|----------|-----------|
| North half | $(4.5 \pm 0.3) \times 10^{-4}$                       | $(8.0 \pm 1.0) \times 10^{-4}$                       | $(1.08 \pm 0.23)$                                  | 222      | 193       |
| South half | $(4.4 \pm 0.8) \times 10^{-4}$                       | $(11.3 \pm 1.0) \times 10^{-4}$                      | $(1.03 \pm 0.26)$                                  | 205      | 189       |



FIG. 3 Pulse-height spectra for the on-cloud and off-cloud diffuse emission; at energies below  $\sim 0.5$  keV, the on-cloud counts are markedly lower than the off-cloud points. The on-cloud spectrum was taken from inside the  $2 \text{ MJy sr}^{-1}$  contour of the  $100\text{-}\mu\text{m}$  map. Spectral fits were performed between 0.09 and 2.48 keV.



with the presence of a considerable molecular component for this cloud<sup>14</sup>.

The energy dependence of the Draco shadow is shown in Fig. 3, which compares X-ray pulse-height spectra for the cloud and the surrounding background region. We subtracted the off-cloud from the on-cloud spectra to obtain spectra of the distant component, which were fitted to single-temperature thermal models (J. C. Raymond and B. W. Smith, personal communication as update to ref. 19). The best-fit parameters for the northern half of the field are  $N_{\text{H}} = 0.69 \times 10^{20} \text{ cm}^{-2}$ , emission measure =  $0.0063 \text{ cm}^{-6} \text{ pc}$  and  $\log T = 6.07$ ; the southern half has  $N_{\text{H}} = 0.16 \times 10^{20} \text{ cm}^{-2}$ , emission measure =  $0.0058$  and  $\log T = 6.14$ . These values are consistent with the conclusions drawn from

the fits to equation (1), but the spectral parameters are poorly constrained.

These data are consistent with a simple model for this line of sight. Emission from the Local Bubble accounts for most of the  $\frac{1}{4}$  keV flux directly toward the Draco cloud. The Draco cloud shadows the remainder of the  $\frac{1}{4}$  keV background emission, which therefore must originate high above the galactic plane. The distant component also experiences foreground absorption by low-velocity neutral gas located outside the Local Bubble, and a gradient in the column density of this foreground gas produces a gradient in the soft X-ray intensity across our image. The bulk of the  $\frac{1}{4}$  keV emission seems to be located between us and the Draco cloud.

These data provide the first firm observational evidence for the presence of million-degree gas in the galactic halo. The presence of such gas in the halo has long been postulated on theoretical grounds<sup>20–22</sup> and has also been used as an explanation for the global anticorrelation between  $N_{\text{H}}$  and the  $\frac{1}{4}$  keV diffuse background<sup>4</sup>. But the distant X-ray component observed towards the Draco cloud cannot explain this global anticorrelation without conflicting with previous failures to find shadowing by high-latitude gas<sup>1–3</sup>. Furthermore, the effective absorption cross-section found here is inconsistent with that found in fitting global absorption models. We conclude that the shadow is cast by a localized hot spot in the galactic halo, perhaps associated with a supernova remnant blowout from the disk. We do not believe, however, that any generalizations can be drawn from these data to the rest of the sky until similar observations have been performed for a wide variety of absorbing clouds.  $\square$

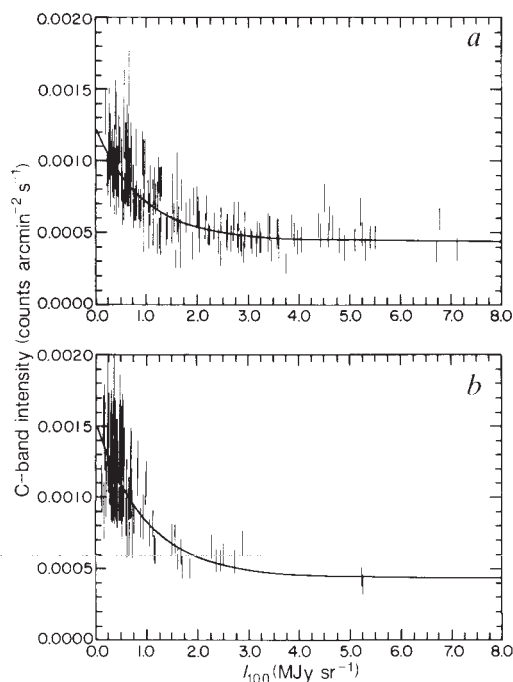


FIG. 2 Fit of the two-component slab absorption model (equation (1)) to our C-band data. The unsmoothed  $100\text{-}\mu\text{m}$  and diffuse X-ray maps were binned into  $5' \times 5'$  pixels, and a systematic uncertainty of 10% was added to account for small-scale fluctuations (see Fig. 1) which are not included in our simple model. Because the off-cloud C-band rate varies across the image, we performed the fits separately for the northern and southern halves of the image. Model parameters are given in Table 1. a, Absorption model fit for the northern half of our field. b, Fit for the southern half of our field.

Received 21 April; accepted 22 May 1991.

- McCammon, D., Meyer, S. S., Sanders, W. T. & Williamson, F. O. *Astrophys. J.* **209**, 46–52 (1976).
- Long, K. S., Agrawal, P. C. & Garmire, P. C. *Astrophys. J.* **206**, 411–417 (1976).
- Burrows, D. N., McCammon, D., Sanders, W. T. & Kraushaar, W. L. *Astrophys. J.* **287**, 208–218 (1984).
- Marshall, F. J. & Clark, G. W. *Astrophys. J.* **287**, 633–652 (1984).
- Jakobsen, P. & Kahn, S. M. *Astrophys. J.* **309**, 682–693 (1986).
- Snowden, S. L., Cox, D. P., McCammon, D. & Sanders, W. T. *Astrophys. J.* **354**, 211–219 (1990).
- McCammon, D., Bunner, A. N., Coleman, P. L. & Kraushaar, W. L. *Astrophys. J.* **168**, L33–L37 (1971).
- Trümper, J. *Adv. Space Res.* **2**, 241–249 (1983).
- Snowden, S. L., Mebold, U., Hirth, W., Herbstmeier, U. & Schmitt, J. H. M. M. *Science* (in the press).
- Goerigk, W., Mebold, U., Reif, K., Kalberla, P. M. W. & Velden, L. *Astr. Astrophys.* **120**, 63–73 (1983).
- Odenwald, S. F. *Astrophys. J.* **325**, 320–341 (1988).
- Lilienthal, D., Wennmacher, A., Herbstmeier, U. & Mebold, U. *Astr. Astrophys.* (in the press).
- Magnani, L., Blitz, L. & Mundy, L. *Astrophys. J.* **295**, 403–421 (1985).
- Mebold, U. *et al. Astr. Astrophys.* **151**, 427–434 (1985).
- Boulanger, F. & Perault, M. *Astrophys. J.* **330**, 964–985 (1988).
- Bowyer, C. S., Field, G. B. & Mack, J. E. *Nature*, **217**, 32–34 (1968).
- Jahoda, K., McCammon, D., Dickey, J. M. & Lockman, F. J. *Astrophys. J.* **290**, 229–237 (1985).
- Jahoda, K., McCammon, D. & Lockman, F. J. *Astrophys. J.* **311**, L57–L61 (1986).
- Raymond, J. C. & Smith, B. W. *Astrophys. J. suppl. Ser.* **35**, 419–439 (1977).
- Spitzer, L. *Astrophys. J.* **124**, 20 (1956).
- Chevalier, R. A. & Oegerle, W. R. *Astrophys. J.* **227**, 398–406 (1979).
- Bregman, J. N. *Astrophys. J.* **236**, 577–591 (1980).

# Structure of single-phase superconducting $K_3C_{60}$

Peter W. Stephens\*, Laszlo Mihaly\*, Peter L. Lee†, Robert L. Whetten‡, Shiou-Mei Huang‡, Richard Kaner‡, François Deiderich‡ & Karoly Holczer‡

\* Department of Physics, State University of New York, Stony Brook, New York, 11794, USA

† New York State Institute on Superconductivity and Department of Chemistry, State University of New York, Buffalo, New York 14214, USA

‡ Department of Physics, Department of Chemistry and Biochemistry, and Solid State Science Center, University of California, Los Angeles, California 90024-1569, USA

**RECENT reports<sup>1-3</sup> of superconductivity in alkali-metal-doped compounds of the icosahedral  $C_{60}$  (buckminsterfullerene) molecule have attracted great experimental and theoretical interest. Superconductivity was originally discovered in samples prepared from gas-solid reactions<sup>1</sup>, which made it impossible to determine the composition or structure of the superconducting phase. Holczer *et al.*<sup>4</sup> demonstrated that potassium-doped  $C_{60}$  has only a single stable superconducting phase,  $K_3C_{60}$ , with a transition temperature of 19.3 K. Improvements have since resulted in the preparation of 100% bulk superconductors<sup>3</sup>. Because of the absence of impurity phases, we have been able to perform accurate Rietveld analysis of X-ray diffraction data from the superconducting phase. Here we report our results for the crystal structure of  $K_3C_{60}$ , determining that this superconducting compound has a face-centred cubic structure with a well defined stoichiometry. These results should open the way to rigorous description of the normal and superconducting properties of this compound.**

The structures that have been determined so far include those of the pure, electrically insulating  $C_{60}$  (ref. 5) and the heavily doped  $K_6C_{60}$  (ref. 6). At room temperature, solid  $C_{60}$  forms a face-centred cubic (f.c.c.) lattice with 10.0 Å intercluster separation<sup>5</sup>.  $K_6C_{60}$  has a body-centred cubic structure, with K atoms in distorted tetrahedral sites<sup>6</sup>. No superconductivity was observed in that material.  $Cs_8C_{60}$  has the same structure<sup>6</sup>. We have found that the superconductor  $K_3C_{60}$  has a f.c.c. structure derived from that of  $C_{60}$  by incorporating K ions into all of the octahedral and tetrahedral interstices of the host lattice.

Preparation of these powder samples has been described previously<sup>2</sup>. Briefly, the reaction between stoichiometric

TABLE 1 Structural parameters from Rietveld analysis for  $K_3C_{60}$

|    | Site |   | x     | y     | z     | Occupancy  | $B$ (Å <sup>2</sup> ) |
|----|------|---|-------|-------|-------|------------|-----------------------|
| C1 | 96   | j | 0     | 0.046 | 0.245 | 50%        | 1.2 ± 0.5             |
| C2 | 192  | l | 0.213 | 0.084 | 0.096 | 50%        | 1.2 ± 0.5             |
| C3 | 192  | l | 0.184 | 0.160 | 0.051 | 50%        | 1.2 ± 0.5             |
| K1 | 8    | c | 1/4   | 1/4   | 1/4   | 100% ± 20% | 6.5 ± 2               |
| K2 | 4    | b | 1/2   | 1/2   | 1/2   | 100% ± 10% | 16 ± 6                |

Crystallographic space group is  $Fm\bar{3}m$  ( $O_h^5$ ). Cubic lattice parameter  $a = 14.24 \pm 0.01$  Å.  $R$  values for fit are  $R_{wp} = 8\%$ ,  $R_1 = 17\%$ .

amounts of solid  $C_{60}$  and K vapour proceeds in several stages, until a material that is very rich in the superconducting phase is achieved. Powder samples of 50% or higher diamagnetic shielding at 4.2 K are produced. The superconducting ceramic pellets are made by one or more cycles of pressing, sintering and grinding<sup>4</sup>.

Figure 1 shows the temperature dependence of the diamagnetic shielding. Data were taken using a standard magnetic inductance coil bridge, operated at 100 kHz; the diamagnetic fraction is expressed relative to a niobium standard. Within the precision of the instrument, 100% diamagnetism is achieved for the ceramic sample at temperatures below 10 K, indicating that the magnetic field is completely expelled from the bulk of the sample. Apart from the onset temperature at 19.3 K, the curve shows a kink and a sharp decline below 17.5 K. As discussed elsewhere<sup>4</sup>, such features may be interpreted in terms of weak links between superconducting grains.

A number of pressed samples, of volume  $\sim 1$  mm<sup>3</sup>, were cut from pellets and sealed in pyrex capillaries for powder diffraction measurements. Before the X-ray studies, the diamagnetic fraction was measured and found to be close to 100%. The samples were never removed from the capillaries, so we can be confident that they did not degrade from exposure to air. In addition to the detailed spectrum presented below, we observed essentially identical diffraction patterns from a total of five samples from three separate batches.

Powder diffraction spectra were collected at the State University of New York X3A beamline at the National Synchrotron Light Source. X-rays of wavelength  $\lambda = 0.800$  Å were produced by a double Si(111) monochromator, and the diffracted beam was analysed with a Ge(111) crystal. The resulting resolution was  $0.05^\circ$  full width at half maximum. The room-temperature diffraction pattern is shown in Fig. 2. For comparison, we also show the powder diffraction pattern of pure  $C_{60}$  taken at  $\lambda = 1.500$  Å. The  $C_{60}$  spectrum matches previous results<sup>5</sup>.

Both the  $C_{60}$  and  $K_3C_{60}$  patterns correspond to f.c.c. lattices of essentially identical unit-cell size: 14.11 Å for  $C_{60}$  and 14.24 Å for  $K_3C_{60}$ . The full width at half maximum of the strongest reflections in the  $K_3C_{60}$  sample is  $0.1^\circ$ , corresponding to a grain size of at least 500 Å. The higher background scattering in this sample is a consequence of its heavier capillary; any amorphous background scattering is no stronger than for the pure  $C_{60}$  material.

As both samples have a f.c.c. structure of equal unit-cell dimensions, it is apparent that the  $K_3C_{60}$  structure is based on a f.c.c. lattice of  $C_{60}$  molecules, with K atoms inserted into empty spaces. The possibility of incorporating alkali ions into these sites was noted previously by Haddon *et al.*<sup>7</sup>. The clearest difference between the  $C_{60}$  and the  $K_3C_{60}$  diffraction patterns occurs in the relative intensities of the strongest few peaks, most notably (220) and (311). It is therefore evident that the sites occupied by K interfere destructively and constructively with the C atoms, respectively, for these two reflections. This immediately suggests that the K occupy sites such as  $(\frac{1}{4}, \frac{1}{4}, \frac{1}{4})$  or  $(\frac{1}{2}, 0, 0)$ , shown in Fig. 3. The fact that the stoichiometry

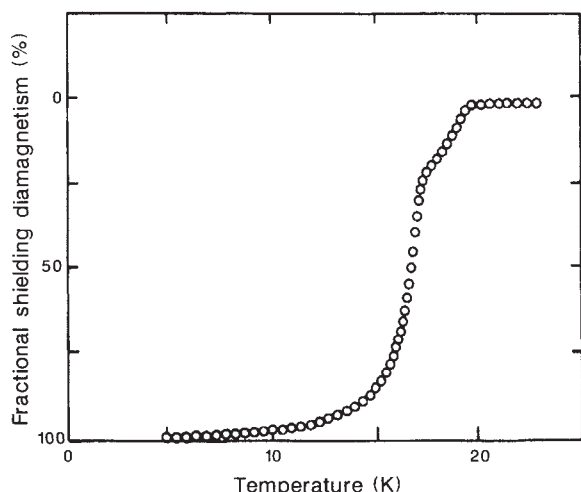


FIG. 1 Diamagnetic shielding signal against temperature for the sample of  $K_3C_{60}$  used in the detailed X-ray study.



requires three K atoms for each  $C_{60}$  molecule implies that K atoms occupy both of these inequivalent sites. The structure is essentially the well known cryolite structure, which applies to a large class of ionic solids<sup>8</sup>.

An important issue in describing this structure is the orientational order of the  $C_{60}$  molecules. Consideration of the size of the constituents severely limits the possible choices. Free rotation of the molecules, as is observed in pure  $C_{60}$ , can be immediately ruled out. The shortest distance between a K atom at the  $(\frac{1}{4}, \frac{1}{4}, \frac{1}{4})$  position and C atom on the freely rotating  $C_{60}$  would be 2.66 Å, much smaller than the 3.06 Å measured in intercalated graphite<sup>9</sup>. The estimate by Haddon *et al.*<sup>7</sup> also suggests that the molecules are locked into a fixed position: a rotating (spherical)  $C_{60}$  unit leaves a tetrahedral site of radius 1.12 Å, whereas the radius of a  $K^+$  ion is 1.33 Å. In fact, there are only two possible orientations of the  $C_{60}$  molecule that provide sufficient space for all eight nearest-neighbour alkali-metal atoms. In both cases the  $C_{60}$  molecule is oriented with eight of its twenty hexagonal faces along the cubic  $\langle 111 \rangle$  directions. The two orientations are related by a 90° rotation about the (100) direction, producing different positions for the pentagonal faces, as shown in Fig. 3.

We performed a Rietveld refinement of the  $K_3C_{60}$  powder diffraction pattern for two structures allowed by these size restrictions. In the first case, we assumed that all  $C_{60}$  units have the same orientation. The space group is then  $Fm\bar{3}$ , and we find that the refinement always yields a significant peak at  $3.3 \text{ Å}^{-1}$ , which is not seen in the experiment. In the second case, the molecules are disordered between the two possible orientations, as illustrated in Fig. 3. The fit shown in Fig. 2 is in excellent agreement with the data. Table 1 summarizes the fitting parameters. The space group is  $Fm\bar{3}m$ , which is a higher symmetry

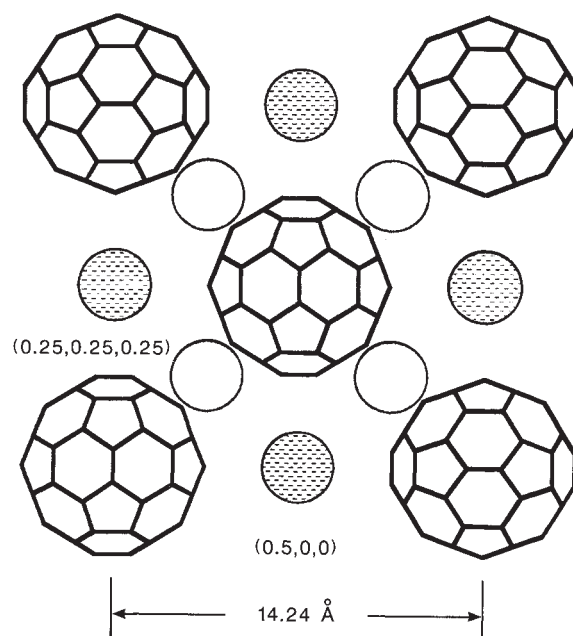


FIG. 3 The structure of  $K_3C_{60}$ . The open and hatched spheres represent the potassium at the tetrahedral and octahedral sites, respectively.

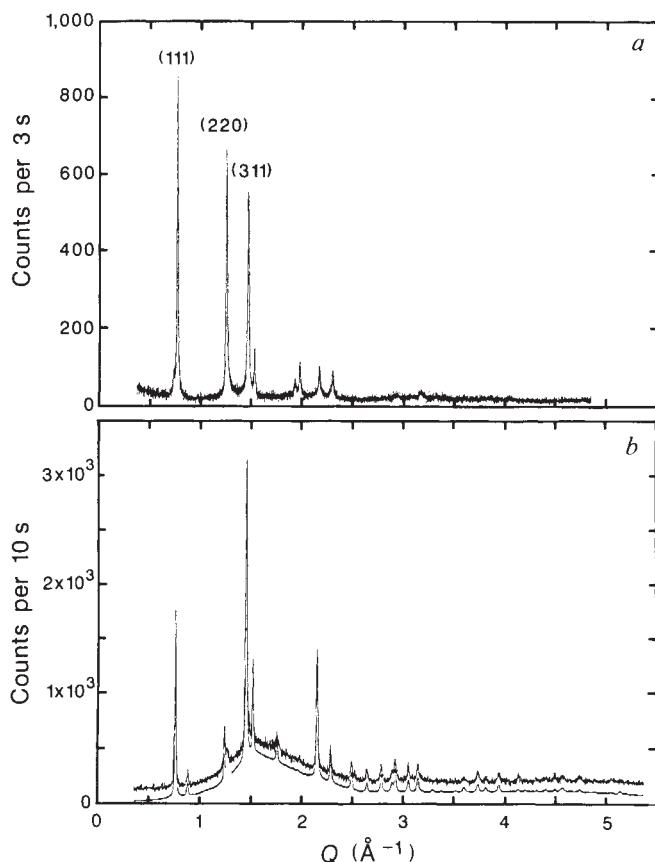


FIG. 2 X-ray diffractogram of *a*, the pure  $C_{60}$  and *b*, the  $K_3C_{60}$  compounds. The Rietveld fit, shown for the  $K_3C_{60}$ , is shifted for clarity.

than allowed for the icosahedral molecule, but can be interpreted as a 50% occupancy of the two orientations. X-ray diffraction cannot distinguish between dynamic or frozen disorder.

We find the radius of the  $C_{60}$  molecule to be 3.54 Å, in satisfactory agreement with values derived from undoped  $C_{60}$ . The carbon atoms move from their ideal positions by at most 0.02 Å, a small but significant distortion. The temperature factors are strongly correlated with the background correction, but two points can be made: first, *B* factors for the carbon atoms are small enough to confirm that there is little rotation of the molecules, and second, the large thermal factor for the octahedral cation reflects the larger space available for it.

The nearest-neighbour C-K distance in this structure is 3.27 Å, separating the potassium atoms at the  $(\frac{1}{4}, \frac{1}{4}, \frac{1}{4})$  tetrahedral site from the six-membered carbon rings directly facing them in each of four  $C_{60}$  molecules. The distance from the octahedral K site at  $(\frac{1}{2}, 0, 0)$  to the nearest C atom (at  $(0, 0.0501, 0.243)$ ) is 3.69 Å. The closest K-K distance is 6.17 Å, much larger than in metallic potassium.

The determination of the structure is of great interest in connection with other known properties of superconducting  $K_3C_{60}$ . Our observation of orientational disorder and the large thermal factor for K suggests some interesting possibilities. The most important feature of the structure determined here is that it is so similar to that of the undoped solid. Pure  $C_{60}$  is a molecular insulator with no partially filled electronic bands, whereas the doped compound is a metal. As there is no significant change in the lattice spacing, it is possible to assume that the doping changes the filling of the band while leaving the band structure unchanged. Because the bands are formed by weak overlap of molecular orbitals, they are expected to be narrow<sup>10</sup>. Magnetization measurements on a nearly optimized powder sample with  $T_c = 19.3 \text{ K}$  indicate that  $K_3C_{60}$  is an extreme type II superconductor with a London penetration depth of 2,400 Å and a coherence length of 26 Å (ref. 11). These parameters indicate that a high density of states builds up in a narrow energy range around the Fermi level. The observed strong decrease of  $T_c$  with pressure (near  $-0.8 \text{ K kbar}^{-1}$ ) further confirms this picture<sup>4</sup>.

The successful Rietveld fit of the data, in which the K ions occupy all of the available sites in a virtually unchanged  $C_{60}$  lattice, is a direct experimental indication of the microscopic

homogeneity of a material that has a stoichiometric K:C<sub>60</sub> ratio of 3:1. The presence of two crystallographically different K sites suggests that it may be possible to derive other stable compounds. It remains to be seen if these possibilities can be realized by doping with potassium or with other elements, such as rubidium, known to lead to superconductivity at even higher temperatures<sup>2,3</sup>. □

Received 31 May; accepted 5 June 1991.

1. Hebard, A. F. *et al.* *Nature* **350**, 600–601 (1991).
2. Holczer, K. *et al.* *Science* **252**, 1154 (1991).
3. Rosselsky, M. J. *et al.* *Phys. Rev. Lett.* **66**, 2830–2832 (1991).
4. Sparr, G. *et al.* *Science* (submitted).
5. Heiney, P. A. *et al.* *Phys. Rev. Lett.* **66**, 2911–2914 (1991).
6. Zhou, O. *et al.* *Nature* **351**, 462–464 (1991).
7. Haddon, R. C. *et al.* *Nature* **350**, 320–323 (1991).
8. Bode, H. & Voss, E. *Z. Anorg. Chem.* **290**, 1 (1957).
9. Rudorff, W. *Advances in Inorganic Chemistry and Radiochemistry* Vol. 1 (eds Emelius, H. J. & Sharp, A. G.) 225 (Academic Press, New York, 1959).
10. Weaver, J. H. *et al.* *Phys. Rev. Lett.* **13**, 1741–1744 (1991).
11. Holczer, K. *et al.* *Phys. Rev. Lett.* (submitted).

ACKNOWLEDGEMENTS. S. J. Anz and F. Ertl produced and separated the molecular carbon samples used in this research. We thank G. Gruner for sharing the resources of his laboratory and his NSF grant. Funding was provided by the NSF (P.W.S., L.M., R.L.W., F.N.D. and R.B.K.) and by the David and Lucille Packard Foundation (R.L.W. and R.B.K.). The SUNY beamline at NSLS is supported by the Department of Energy (P.W.S. and P.L.). Part of the research was carried out at the National Synchrotron Light Source, Brookhaven National Laboratory, which is supported by the US Department of Energy, Division of Materials Sciences and Division of Chemical Sciences.

## Solubility and reaction kinetics of solution–solid reactions determined by *in situ* observations

H. Ohmoto\*, K. Hayashi, K. Onuma, K. Tsukamoto, A. Kitakaze, Y. Nakano & Y. Yamamoto

Institute of Mineralogy, Petrology & Economic Geology, Faculty of Science, Tohoku University, Aobayama, Sendai 980, Japan  
\* Department of Geosciences, The Pennsylvania State University, University Park, Pennsylvania 16802, USA

THE solubilities of solid compounds in solution, and the kinetics of the reactions that take place between solid and liquid phases, have usually been studied by indirect methods, which are often time-consuming and can be inaccurate. Here we present a new experimental method, using the titration principle and *in situ* observation of solution–solid reactions on a solid surface, which allows the rapid acquisition of accurate data on the dissolution and growth rates and the solubilities (both stoichiometric and nonstoichiometric) of solids in a variety of aqueous and organic solutions at temperatures up to 350 °C and pressures up to 200 bar. This technique should provide new opportunities for determining the phase diagrams of complex systems and for studying crystal growth.

Conventional methods for determining the solubility of a solid compound in a solution involve reaction of large quantities (typically >1 g) of solid particles and solution in an autoclave at the desired temperature–pressure conditions, withdrawal of the solution and/or solid periodically from the autoclave, and analysis of changes with time in the composition of solution or in the mass of solid<sup>1</sup>. In such methods, the attainment of equilibrium between solid and solution is often difficult to demonstrate, solution and solid compositions may change during quenching, and determination of solution composition, or of change in the mass of solid, with an accuracy of better than ±10% of the true value is often difficult, especially when the solubility is low. In our method, however, neither a chemical analysis of the solution nor weighing the solid after reaction is necessary.

Our method uses the technology developed for *in situ* observation of crystal growth<sup>2,3</sup> and that used for high-temperature hydrothermal experiments of mineral–fluid reactions<sup>1</sup>, along with the principle of titration. A small grain (<1 mg) of a particular solid compound is placed in a specially constructed cell, and reacted with a large mass (>50 g) of solution of precisely known composition. As the reaction proceeds, changes in various surface features (growth or dissolution steps, etch pits and so on) of the solid are observed through a window in the cell using a microscope. The large mass ratio of solution to solid ensures insignificant change in the solution composition as a result of the reaction with the solid. If the solution is undersaturated (or supersaturated) with respect to the solid, some dissolution (or growth) phenomena (such as movement of the growth or dissolution steps, changes in the size and number of etch pits) may be observed on the solid surface. The composition of solution in the cell is then changed systematically by adding aliquots of a titrant; alternatively, the temperature of solution may be changed systematically. *In situ* observation of solid–fluid reaction is continued until no dissolution (or growth) phenomenon is observed (saturation point), or until the dissolution or growth phenomena are reversed. The main differences between our 'titration method' and the normal titration method for determining a solution composition are that, in our method, the solid compound itself, rather than a liquid (such as methyl orange), is used as an indicator for the degree of under- or supersaturation of solution, and that the reaction is monitored through a microscope.

The material and configuration of a reaction cell may vary depending on the type of solid and fluid of interest, and on temperature and pressure conditions of experiments. For example, the cell used for experiments at temperatures below 100 °C and at ~1 bar pressure is made of pyrex glass, has a volume of ~100 cm<sup>3</sup>, and has a small hole for injection of titrants using a micro-syringe. The experimental system for high temperatures and pressures (up to ~350 °C and ~200 bar), illustrated in Fig. 1, has a reaction cell (~1 cm<sup>3</sup> internal volume) and an autoclave (1,000 cm<sup>3</sup> capacity); the solution composition can be adjusted by injecting titrants using high-pressure pumps; the solution is circulated through the system at a rate up to ~50 ml per min by a circulation pump specially designed to handle high-temperature and high-pressure fluids. All parts of the system that come in contact with solutions are made of titanium, except for a sapphire cell window.

The most important component for all our experimental systems is a differential interference-contrast microscope, equipped with a lens aberration corrected for the thicknesses of the window and the solution. This microscope makes it possible to

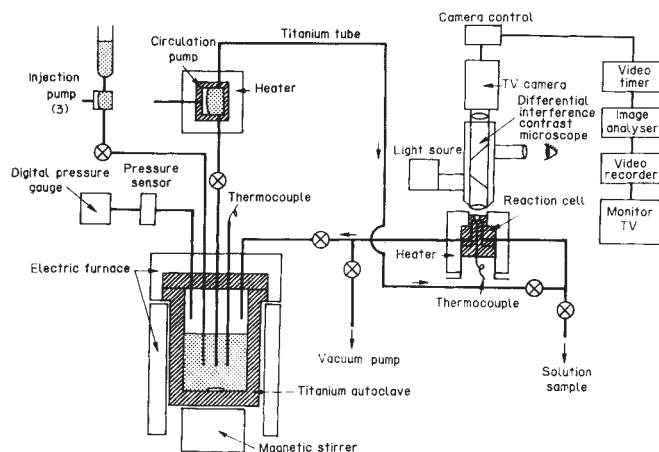


FIG. 1 Schematic diagram of an experimental system for *in situ* observation of solution–solid reactions at temperatures up to 350 °C and pressures up to ~200 bar. The entire system was custom-made by Ko-atsu Kagaku Co. Ltd.



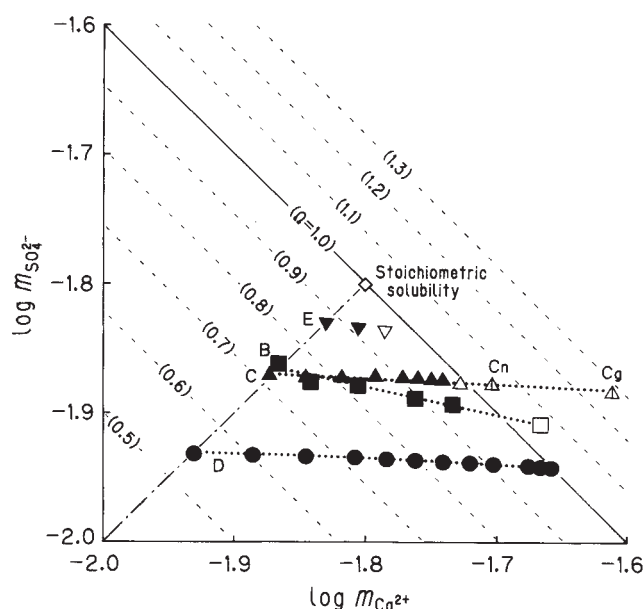


FIG. 2 Relationships between surface dissolution phenomena of a gypsum crystal and the composition (the molalities of  $\text{Ca}^{2+}$  and  $\text{SO}_4^{2-}$ ) of experimental solutions at  $25 \pm 0.1^\circ\text{C}$ . The starting solutions of the four series of experiments (series B–E) lie on the stoichiometric dissolution line ( $m_{\text{Ca}^{2+}} = m_{\text{SO}_4^{2-}}$ ) shown as dot-dashed line; the solution composition was changed successively by addition of  $\text{CaCl}_2$  titrant; experimental solutions within each series are connected by a dotted line. The solid symbols represent the conditions where dissolution phenomena (such as movement of dissolution steps or formation of etch pits) were recognized; the open symbols where no dissolution phenomenon was observed in 12 hours; the two triangle points (Cg and Cn) where growth phenomena were recognized (see text). The solid diagonal line represents saturation ( $\Omega = 1.0$ ), for which  $\log K_{\text{sp}} = \log m_{\text{Ca}^{2+}} + \log m_{\text{SO}_4^{2-}} = -3.60 \pm 0.02$ ; dashed lines are for other values of  $\Omega$  (in brackets).

observe very fine ( $< 1 \mu\text{m}$ ) structural features on the solid surface through layers of aqueous solution several millimetres thick and the cell window (up to 2 mm thick). The images of the surface features, observed through the microscope under reflected (rather than transmitted) light for transparent solids as well as for opaque ones, are recorded continuously by a high-resolution video system, or periodically by a photographic camera, depending on the time required for an experiment.

Dissolution (or growth) of many solid compounds involves two or more species in solution. For example, dissolution (or growth) of gypsum ( $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ ) in aqueous solution involves  $\text{Ca}^{2+}$  and  $\text{SO}_4^{2-}$  ions:



For such systems, therefore, the titration can be carried out using either solution species as the titrant. In our study at  $25^\circ\text{C}$ , four types of starting solutions, having equal molar amounts of  $\text{Ca}^{2+}$  and  $\text{SO}_4^{2-}$  but different total concentrations, were prepared by dissolving appropriate amounts of reagent-grade  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$  powder in distilled water. Two titrant solutions containing 0.10 and 0.47 mol per kg  $\text{H}_2\text{O}$  of  $\text{CaCl}_2$  were also prepared. A small cleaved gypsum crystal ( $\sim 2 \times 2 \times 1 \text{ mm}$ ) was mounted inside the reaction cell and reacted with one of the starting solutions in the reaction cell for 30–60 minutes. During this period, changes in the surface features (etch pits, cleaved steps) of the gypsum crystal were continuously observed using the video system. The solution composition was then changed by adding  $0.2\text{--}0.5 \text{ cm}^3$  aliquots of one of the titrants; observation of the crystal surface continued for 30–60 minutes before new titrant was added. When no dissolution phenomenon was observed after 12 hours, this was considered an 'end point' of the reaction (see later for justification).

The concentrations of  $\text{Ca}^{2+}$  and  $\text{SO}_4^{2-}$  ions in the cell solution at any time during the experiment can be easily computed from their concentrations in the starting solution and in the titrant, and the proportions of the starting solution and the titrant in the cell (see Fig. 2). When the experimental solutions and gypsum attain equilibrium (that is, the solutions are saturated with gypsum), the following relationship is satisfied:

$$\begin{aligned} \log K &= \log a_{\text{Ca}^{2+}} + \log a_{\text{SO}_4^{2-}} + 2 \log a_{\text{H}_2\text{O}} \\ &= (\log \gamma_{\text{Ca}^{2+}} + \log \gamma_{\text{SO}_4^{2-}} + 2 \log a_{\text{H}_2\text{O}}) \\ &\quad + \log m_{\text{Ca}^{2+}} + \log m_{\text{SO}_4^{2-}} \end{aligned}$$

where  $a_i$ ,  $m_i$  and  $\gamma_i$  are, respectively, the activity, molality and activity coefficient of a species  $i$ ;  $K$  is the equilibrium constant. As the ionic strengths of the experimental solutions are very similar (between 0.06 and 0.08), the value of  $(\log \gamma_{\text{Ca}^{2+}} + \log \gamma_{\text{SO}_4^{2-}} + 2 \log a_{\text{H}_2\text{O}})$  can be considered constant for all the experimental solutions. Hence, the 'end points' of the titration should lie on a straight line with a slope of  $-1$  on plots of  $\log m_{\text{Ca}^{2+}}$  against  $\log m_{\text{SO}_4^{2-}}$ , defining the equilibrium (or saturation) line. The results of our experiments (Fig. 2) indicate that  $\log K_{\text{sp}} = \log m_{\text{Ca}^{2+}} + \log m_{\text{SO}_4^{2-}} = -3.60 \pm 0.02$  at  $25.0 \pm 0.1^\circ\text{C}$ , where  $K_{\text{sp}}$  is the solubility product of gypsum. The stoichiometric solubility of gypsum, where  $m_{\text{Ca}^{2+}} = m_{\text{SO}_4^{2-}}$ , was determined as  $0.0158 \pm 0.0004 \text{ mol per kg H}_2\text{O}$  at  $25.0^\circ\text{C}$ . In comparison, the values obtained at  $25^\circ\text{C}$  by conventional experimental methods (see refs 4 and 5 for reviews) fall in a range of  $0.0152 \pm 0.0004 \text{ mol per kg H}_2\text{O}$ .

In reactions between gypsum and aqueous solutions, the size and the number of etch pits increase with increasing degree of undersaturation. The dissolution phenomenon that can be most easily quantified, however, is the rate of lateral movement ( $V$ , in units of  $\mu\text{m s}^{-1}$ ) of thin ( $\sim 10\text{--}50\text{-}\mu\text{m}$  thick) cleaved steps in the  $[401]$  direction on the  $(010)$  plane of gypsum (see Fig. 3). When the degree of saturation of solution,  $\Omega$ , is defined as  $\Omega = m_{\text{Ca}^{2+}}(\text{sol})m_{\text{SO}_4^{2-}}(\text{sol})/K_{\text{sp}}$ , our experimental data points lie very close to a single line,  $\ln V = 1.06 \ln (1 - \Omega) + 1.35$ , suggesting that the two-dimensional dissolution of gypsum follows first-order reaction kinetics (H.O. *et al.*, manuscript in preparation).

The above equation yields a value of  $V \approx 0.001 \mu\text{m s}^{-1}$  at  $\Omega = 0.999$ , which implies that the cleaved steps of gypsum should move  $\sim 10 \mu\text{m}$  in 3 hours during reaction with a solution of 99.9% saturation. As no movement of the cleaved steps was

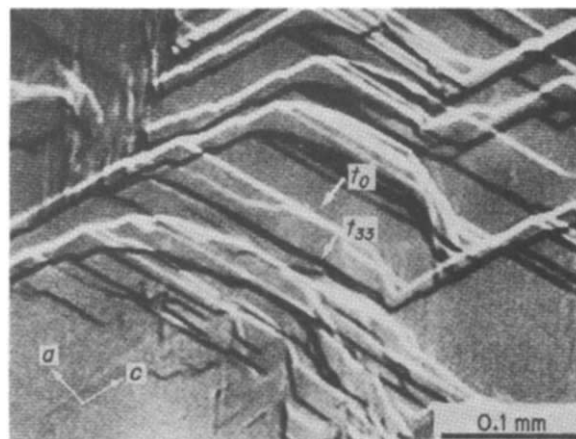


FIG. 3 Composite video picture showing the retreat of the dissolution steps on the  $(010)$  plane of a gypsum crystal during reaction with an undersaturated solution ( $\Omega = 0.96$ ) in 33 minutes. After image-processing the video pictures, the step fronts of the gypsum surface at time 0 ( $t_0$ ) are shown by white lines, and those after 33 minutes ( $t_{33}$ ) by black lines,  $a$  and  $c$  represent the two crystallographic axes. The NW–SE lines represent the  $[401]$  dissolution steps, which retreated in the  $[102]$  direction.

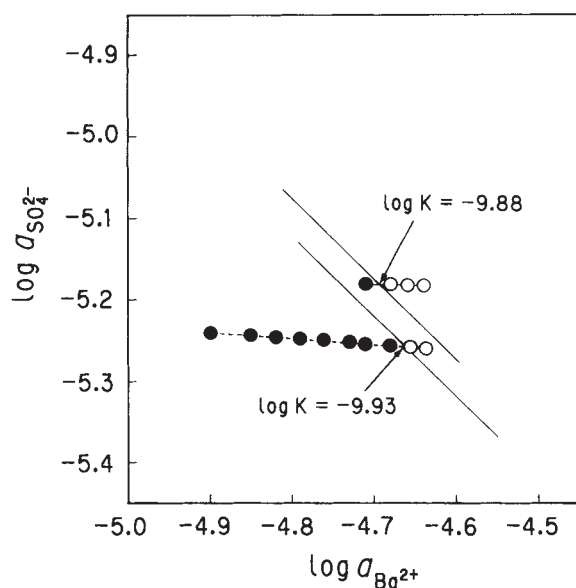


FIG. 4 Relationships between surface dissolution phenomena of a barite crystal and the composition (the activities of  $\text{Ba}^{2+}$  and  $\text{SO}_4^{2-}$ ) of experimental solutions containing 3M NaCl at  $T = 150 \pm 0.5^\circ\text{C}$ . The solution composition was changed successively by addition of  $\text{BaCl}_2$  titrant. The activity values for  $\text{Ba}^{2+}$  and  $\text{SO}_4^{2-}$  were computed from the molalities of  $\Sigma\text{Ba}$ ,  $\Sigma\text{SO}_4^{2-}$ ,  $\Sigma\text{Na}$ , and  $\Sigma\text{Cl}$  of the experimental solutions using the extended Debye-Hückel method. The solid symbols represent the conditions where dissolution phenomena were recognized; the open symbols where no dissolution phenomenon was observed in 6 hours. Solid lines represent two values of  $\log K$  where  $K = a_{\text{Ba}^{2+}} a_{\text{SO}_4^{2-}}$ .

observed in 12 hours at the 'end points' of the titration, the  $\Omega$  values of the 'end-point' solutions must be greater than 0.999, and possibly greater than 1.0 (supersaturation). Reversal of the dissolution process (in other words, crystal growth) using the same crystal was not detected until the  $\Omega$  value exceeded 1.26 (see point Cg in Fig. 2), probably because many of the crystal defects, which could become nucleation sites, had been dissolved away during the earlier reactions with undersaturated solutions. When the 'old' gypsum crystal was replaced by a 'fresh' one at the saturation point and the titration was continued, some crystal growth features were recognized at  $\Omega \geq 1.06$  (see point Cn in Fig. 2). These experiments indicate that the end points (equilibrium values) of solution-solid reactions can be determined much more precisely by observing dissolution rather than growth phenomena of crystals.

Figure 4 is an example of results from our experiments on dissolution of barite in a 3M NaCl solution at  $150^\circ\text{C}$  using the experimental system shown in Fig. 1. The equilibrium constant for dissociation of barite,  $\log K = \log a_{\text{Ba}^{2+}} + \log a_{\text{SO}_4^{2-}}$ , is found to be  $-9.90 \pm 0.03$  at  $150^\circ\text{C}$  from our two series of experiments (K.H. *et al.*, manuscript in preparation). In comparison, the  $\log K$  value at  $150^\circ\text{C}$  based on the experimental data of Blount<sup>6</sup> on the solubility of barite in NaCl solutions and on more recent thermodynamic data (see ref. 7 for summary) for the aqueous species ranges from  $-9.5$  to  $-10.2$ . Blount<sup>6</sup> required run times of several weeks to demonstrate equilibrium between barite and aqueous solution at temperatures around  $150^\circ\text{C}$ , and the experimental solutions had to be analysed for barium and/or sulphate. In contrast, our two series of barite experiments (Fig. 4) were completed in less than 2 days, and a chemical analysis of the experimental solution was not necessary, demonstrating some of the advantages of our experimental method.

Barite is one of the least soluble minerals in nature; its solubility is about three orders of magnitude lower than that of gypsum at room temperature<sup>4,5</sup>. The two examples given (gypsum at  $25^\circ\text{C}$  and barite at  $150^\circ\text{C}$ ) illustrate that the method

described here is applicable to studies aimed at obtaining precise data on the solubility and reaction kinetics of a wide variety of solution-solid reactions, including those with very low solubility, over wide ranges of temperature and pressure. □

Received 23 January; accepted 29 April 1991.

1. Ulmer, G. C. & Barnes, H. L. (eds) *Hydrothermal Experimental Techniques* (Wiley, New York, 1987).
2. Tsukamoto, K. *J. Cryst. Growth* **61**, 199–209 (1983).
3. Tsukamoto, K. & Sunagawa, I. *J. Cryst. Growth* **71**, 183–190 (1985).
4. Blount, C. W. & Dickson, F. W. *Am. Miner.* **58**, 323–331 (1973).
5. Holland, H. D. & Malinin, S. D. in *Geochemistry of Hydrothermal Ore Deposits* 2nd ed (ed. Barnes, H. L.) 461–508 (Wiley, New York, 1979).
6. Blount, C. W. *Am. Miner.* **62**, 942–957 (1977).
7. Ohmoto, H. *et al. Econ. Geol.* **5**, 570–604 (1983).

ACKNOWLEDGEMENTS. We thank M. Matsubara and S. Sugino for technical assistance in the development of the experimental system, and S. L. Brantley, A. C. Lasaga, M. J. Machesky, S. R. Poulson and D. K. Smith for useful comments on an earlier manuscript. This work was supported by the Japanese Ministry of Education, Science and Culture and the NSF.

## Relation between crystal symmetry and ionicity in silica polymorphs

G. J. Kramer\*, B. W. H. van Beest\* & R. A. van Santen\*†

\* Koninklijke/Shell-Laboratorium, Amsterdam (Shell Research B.V.), PO Box 3003, 1003 AA Amsterdam, The Netherlands

† Schuit Institute of Catalysis, Laboratory of Inorganic Chemistry and Catalysis, Eindhoven University of Technology, PO Box 513, 5600 MB Eindhoven, The Netherlands

**THE structure and stability of an inorganic solid is determined in general both by short-range covalent and by long-range electrostatic forces. Here we describe the use of interatomic force fields developed recently from first-principles quantum-chemical cluster calculations<sup>1,2</sup> in the study of the structures of  $\text{SiO}_2$  tetrahedral networks. We find that the symmetry of these structures depends sensitively on the balance between ionic and covalent forces: high-symmetry structures are stabilized for relatively large ion partial charges, and low-symmetry structures are stabilized when the ionicity is small. For some  $\text{SiO}_2$  polymorphs, the low-symmetry structures found in our simulations correspond to the low-temperature phases of these polymorphs found experimentally. A reinterpretation of structural data on quartz provides evidence for temperature dependence of the ionicity, which can explain the change of symmetry observed when temperature is increased. Our preliminary calculations on aluminophosphates suggest that this symmetry-breaking mechanism may also provide insight into the structural changes observed for complex molecular sieves.**

The theoretical study of silicas has advanced to the point where the properties of the simplest structures can be calculated from first principles using quantum chemical tools<sup>3,4</sup>. The study of silicas and silicates with large unit cells, such as zeolites, as well as the study of minerals, has relied on methods that describe the interaction between atoms in these (partially) ionic solids by empirical potentials or force fields; a review is given in ref. 5. Only recently, though, have force-field descriptions based on *ab initio* calculations been proposed for mixed ionic-covalent systems such as silicas<sup>1,2,6,7</sup> and aluminophosphates<sup>1,2</sup>. The advantage of the latter approach over the empirical approach lies in the first-principles description of the silicon-oxygen bond and of the repulsion between neighbouring oxygen atoms. A molecular dynamics study by Tsuneyuki *et al.* employing such a force field has reproduced the various  $\text{SiO}_2$  polymorphs as thermodynamically stable systems<sup>7,8</sup>.

In spite of their foundation in *ab initio* quantum chemistry, force-field methods are ultimately empirical: there is no *a priori*



preferred functional form, and the treatment of the atoms as point-like, charged particles is an abstraction, as a consequence of which the magnitude of the charge is not uniquely defined. This fact, in combination with the infinite range of the electrostatic interactions, precludes the derivation of accurate force fields solely from the potential-energy surface of clusters<sup>1,2</sup>. But the success of force fields based on *ab initio* calculations on small clusters ( $\text{SiO}_4$  or  $\text{Si}(\text{OH})_4$ , refs 7 and 1 respectively) in the prediction of properties directly related to the Si-O bonding, such as the vibrational frequencies of quartz<sup>2</sup>, does indicate that the covalent bonding is correctly described by such methods. Here we will exploit this fact and, at the same time, use the indeterminacy of Coulomb interactions to study the effect of changes in the balance between covalent and ionic interactions on the structure of  $\text{SiO}_2$  polymorphs, in particular with respect to crystal symmetry.

We have performed a series of lattice-energy-minimization calculations on each of five  $\text{SiO}_2$  polymorphs (quartz, cristobalite, coesite, stishovite and silicalite) using force fields, based on first principles, which differ in ionicity. The effective charge on silicon ( $Q_{\text{Si}}$ ) is varied between 2 and 3 (at 0.1 or 0.2 intervals), and is thus significantly smaller than the formal silicon charge of 4, thereby complying with the mixed ionic-covalent character of the system. For each choice of  $Q_{\text{Si}}$ , parameters of the short-range force field have been determined that fit the potential energy surface of the  $\text{Si}(\text{OH})_4$  molecule. This ensures the correct description of the covalent interactions within the  $\text{SiO}_4$  tetrahedron for all choices of  $Q_{\text{Si}}$ .

The crystal symmetry of the systems as modelled can be read from the calculated unit-cell matrix and from the elasticity matrix<sup>9</sup> or the bulk modulus. Figure 1 shows the dependence of the calculated bulk modulus on  $Q_{\text{Si}}$ . Between  $Q_{\text{Si}} = 2.4$  and  $Q_{\text{Si}} = 2.6$ , the bulk moduli of quartz, cristobalite and silicalite (the all-silica form of zeolite ZSM-5) (ref. 10) show a sudden change, which reflects a change of symmetry. For quartz, the low- $Q_{\text{Si}}$  phase corresponds to low quartz or  $\alpha$ -quartz; the high- $Q_{\text{Si}}$  phase is high quartz or  $\beta$ -quartz. For cristobalite the same symmetry breaking occurs, and for silicalite the high- $Q_{\text{Si}}$  struc-

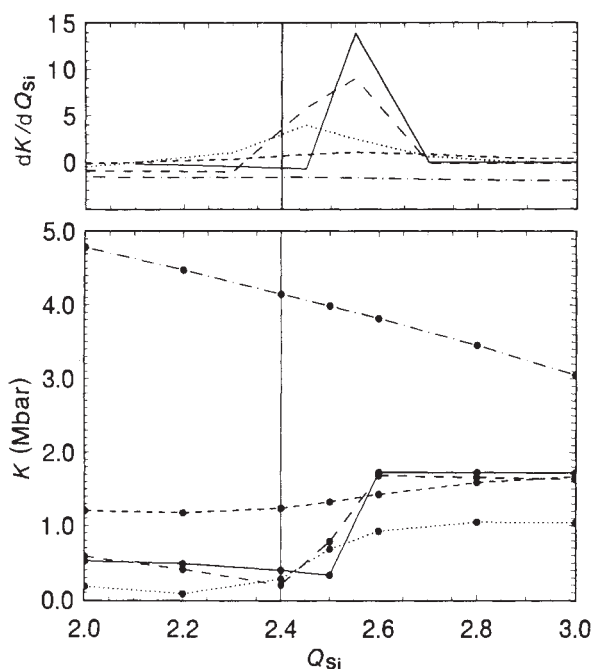


FIG. 1 Calculated bulk moduli ( $K$ ) plotted against silicon charge for quartz (—), cristobalite (---), coesite (.....), silicalite (— · — · —) and stishovite (— · — · —). The force field with  $Q_{\text{Si}} = 2.4$  gives the best description of the low-temperature structure and elastic properties of  $\alpha$ -quartz. The transitions from low to high symmetry occur roughly at  $Q_{\text{Si}} = 2.5$ , as can be seen clearly from the numerical derivatives ( $dK/dQ_{\text{Si}}$ ).

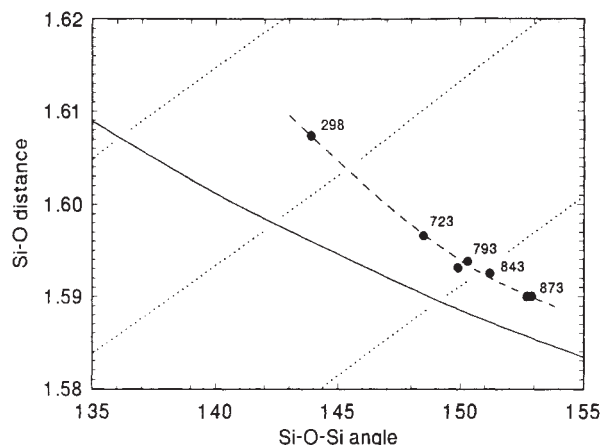


FIG. 2 Experimental relation between Si-O distance and Si-O-Si angle for quartz at different temperatures (circles; the dashed line is a guide to the eye), compared with the theoretical prediction as obtained from ST03G calculations on the disilanol molecule (solid line). The dotted curves are lines of equal Mulliken charge (top left, 1.35, to bottom right, 1.37).

ture is orthorhombic and the low- $Q_{\text{Si}}$  structure triclinic. The symmetries of the energy-minimized structures of coesite and stishovite are not affected.

These computer simulations bear an intriguing resemblance to the actual crystal symmetry found in nature. The low- $Q_{\text{Si}}$  phases correspond to the low-temperature phases of silica ( $\alpha$ -quartz, low cristobalite and monoclinic silicalite), and the high- $Q_{\text{Si}}$  values correspond to the high-temperature phases  $\beta$ -quartz ( $T > 853$  K; ref. 11), high-cristobalite ( $T > 493$  K; ref. 12) and orthorhombic silicalite ( $T > 628$  K; ref. 13). Coesite and stishovite, for which no structural phase transitions are known, do not display any ionicity-induced changes in our simulations.

This striking similarity suggests a coupling between the ionicity of silicas and temperature. Evidence for such a coupling can be found from a combination of experimental information on quartz and *ab initio* calculations on small clusters.

The crystallographic study of quartz at elevated temperatures<sup>14</sup> has revealed that, as the temperature is raised from 293 to 873 K, the Si-O-Si angle ( $\theta$ ) increases steadily from 144 to 153°. Such an increase is to be expected because the low-energy phonons of quartz (or any other silica) have primarily a bond-bending character<sup>15</sup>. Coupled to the increase in bonding angle, the bond distance decreases from 1.607 to 1.590 Å (see Fig. 2). This negative coupling is the result of the changing hybridization on the oxygen atoms. *Ab initio* calculations on disilanol ( $\text{H}_6\text{Si}_2\text{O}_7$ )<sup>16</sup> reveal a low-energy valley running diagonally across the (bond-angle, bond-length) (Fig. 2). Along this valley, the ionicity of the disilane molecule increases with the bonding angle. This is seen from changes in the charge on silicon, as calculated from the occupation of the silicon atomic orbitals (Mulliken charge), which increases by  $\sim 0.02e$ , where  $e$  is the charge on an electron, on silicon. This effect will be amplified in bulk silica, where all Si-O-Si angles around silicon are increased, instead of just one, as in the disilanol cluster. Therefore, in bulk silica the change in effective charge on silicon may be as large as  $0.1e$ . This value is roughly equal to the difference (in  $Q_{\text{Si}}$ ) between the ionicity of the low-temperature force field ( $Q_{\text{Si}} = 2.4$ ) and the phase boundary between low- and high-symmetry structures ( $Q_{\text{Si}} \approx 2.55$ ). It is important that both ionicity differences are of the same order of magnitude. Numerical correspondence is not expected, as the definitions of charge in the molecular calculations and in the force-field calculations differ, demonstrating the ambiguity in the definition of point charges mentioned earlier.

As force-field modelling of quartz<sup>1,2</sup> has shown that the balance between ionic and covalent forces of the force field that

best describes  $\alpha$ -quartz is very close to a transition to  $\beta$ -quartz, we conclude that the high-temperature,  $\beta$  phase of quartz is an 'ionicity-stabilized' phase. This conclusion contrasts with the findings of a molecular-dynamics study by Tsuneyuki *et al.*<sup>17</sup>, who conclude that  $\beta$ -quartz is a dynamic phase, oscillating in between the two chirally opposite realizations of  $\alpha$ -quartz symmetry. In the latter picture it is difficult to explain the existence of an intermediate, incommensurate phase of quartz in an interval of 1.8 K near the transition temperature<sup>18</sup>. A Landau-type theory<sup>19</sup> gives a proper description of the  $\beta$ -to-incommensurate transition, explicitly requiring a  $\beta$  phase characterized by a single minimum.

The striking similarity between the transitions in quartz, cristobalite and zeolite MFI, both in simulation and in the observed character of the transition, leads us to conclude that the phase transitions have the same origin: an increased ionicity of the system induced by the increased angle of bonding on oxygen.

Here we are dealing with subtle changes in the crystal symmetry, but it is worth noting that a correspondence between ionicity and atomic coordination has been found previously. As

pointed out by Phillips<sup>20</sup>, binary semiconductors show a change from fourfold to sixfold coordination with increasing ionicity.

We have carried our simulation one step further, to the study of aluminophosphates (ALPOs). In our simulations we can reproduce the  $\alpha$ -to- $\beta$  transition in berlinite (ALPO quartz), which is analogous to that in quartz, by increasing the relative strength of electrostatic interactions in the ALPO system. Moreover, analogous transitions occur, for example, in the open, molecular-sieve-type networks of VPI-5 (ref. 24) and ALPO-8. Here, the symmetry breaking has a remarkable geometric consequence: the layers that form the structure shift perpendicular to the crystallographic  $c$  axis, thereby rendering the channels slightly sinusoidal. In both cases, recent structural studies<sup>21–23</sup> have indeed revealed such deformations, which are not present in the idealized network geometries suggested originally<sup>24</sup>.

**Note added in proof.** A recent paper<sup>25</sup> provides experimental evidence that the  $\alpha$ - $\beta$  phase transition in quartz is indeed a structural phase transition, as suggested here, rather than an order-disorder transition as suggested in ref. 17.  $\square$

Received 29 January; accepted 29 April 1991.

1. Van Beest, B. W. H., Kramer, G. J. & Van Santen, R. A. *Phys. Rev. Lett.* **64**, 1955–1958 (1990).
2. Kramer, G. J., Farragher, N. P., Van Beest, B. W. H. & Van Santen, R. A. *Phys. Rev. B* **43**, 5068–5080 (1991).
3. Dovesi, R., Pisani, C., Roetti, C. & Silvi, B. *J. chem. Phys.* **86**, 6967–6971 (1987).
4. Allan, D. C. & Teter, M. P. *Phys. Rev. Lett.* **59**, 1136–1139 (1987).
5. Catlow, R. C. A. & Price, G. D. *Nature* **347**, 243–248 (1990).
6. Lasaga, A. C. & Gibbs, G. V. *Phys. Chem. Miner.* **14**, 107–117 (1987).
7. Tsuneyuki, S., Tsukada, M., Aoki, H. & Matsui, Y. *Phys. Rev. Lett.* **61**, 869–872 (1988).
8. Tsuneyuki, S., Matsui, Y., Aoki, H. & Tsukada, M. *Nature* **339**, 209–211 (1989).
9. Nye, J. F. *Physical Properties of Crystals* (Oxford University Press, Oxford, 1957).
10. Meier, W. M. & Olson, D. H. *Atlas of Zeolite Structure Types*, 2nd edn (Butterworth, Cambridge, 1987).
11. Axe, J. D. & Shirane, G. *Phys. Rev. B* **1**, 342–348 (1970).

12. Peacor, D. R. Z. *Kristallogr.* **138**, 274–298 (1973).
13. Fyfe, C. A., Strobl, H., Kokotailo, G. T., Kennedy, G. J. & Barlow, G. E. *J. Am. chem. Soc.* **110**, 3373–3380 (1988).
14. Grimm, H. & Dörner, B. *J. Phys. Chem. Solids* **36**, 407–413 (1975).
15. Van Santen, R. A. & Vogel, D. L. *Adv. Solid St. Chem.* **1**, 151–224 (1989).
16. Lasaga, A. C. & Gibbs, G. V. *Phys. Chem. Miner.* **16**, 29–41 (1988).
17. Tsuneyuki, S., Aoki, H., Tsukada, M. & Matsui, Y. *Phys. Rev. Lett.* **64**, 776–779 (1990).
18. Gouhara, K. & Kato, N. *J. phys. Soc. Japan* **54**, 1868–1881; 1882–1889 (1985).
19. Mukamel, D. & Walker, M. B. *Phys. Rev. Lett.* **58**, 2559–2562 (1987).
20. Phillips, J. C. *Bonds and Bands in Semiconductors* (Academic, New York, 1973).
21. Dessau, R. M., Schlenker, J. L. & Higgins, J. B. *Zeolites* **10**, 522–524 (1990).
22. Rudolf, P. R. & Crowder, C. E. *Zeolites* **10**, 163–168 (1990).
23. McCusker, L. B., Baerlocher, C., Jahn, E. & Bülow, M. *Zeolites* (in the press).
24. Davis, M. E., Saldarriaga, C., Montes, C., Garces, J. & Crowder, C. *Zeolites* **8**, 362–366 (1988).
25. Tezuka, Y., Shin, S. & Ishigame, M. *Phys. Rev. Lett.* **66**, 2356 (1991).

## NMR evidence for five-coordinated silicon in a silicate glass at atmospheric pressure

Jonathan F. Stebbins

Department of Geology, Stanford University, Stanford, California 94305, USA

**KNOWLEDGE** of the structure of liquid silicates is essential to understanding the properties of materials ranging from magmas in lava flows to melts in glass processing. At 1 atmosphere pressure, a wide range of evidence indicates that most silicon cations in these systems are coordinated by four oxygens in a tetrahedral configuration ( $\text{Si}^{\text{IV}}$ ). Molecular dynamics computer simulations of these liquids have, however, predicted that defect complexes (of relatively low abundance) consisting of silicon with five oxygen neighbours ( $\text{Si}^{\text{V}}$ ) are of key importance in the mechanism by which viscous flow takes place<sup>1–5</sup>. I present here direct experimental evidence from  $^{29}\text{Si}$  NMR studies of  $\text{K}_2\text{Si}_4\text{O}_9$  glass that  $\text{Si}^{\text{V}}$  does exist in silicate liquids at low pressures, and that the abundance of this species increases with temperature, supporting the idea that  $\text{Si}^{\text{V}}$  defects contribute to 'weakening' of the structure of molten silicates.

Pressure-induced changes from  $\text{Si}^{\text{IV}}$  to six-coordinated silicon ( $\text{Si}^{\text{VI}}$ ) are well known in crystalline silicates, and most of the silicon in the deep interior of the earth is probably present in the latter state. Data from  $^{29}\text{Si}$  magic angle spinning (MAS) NMR confirmed the beginning of this transformation in glasses quenched from alkali silicate liquids at pressures up to 12 GPa<sup>6–8</sup>. NMR peaks for  $\text{Si}^{\text{VI}}$  with chemical shifts near to  $-200$  p.p.m. were clearly observed, as seen previously in crystalline silicates

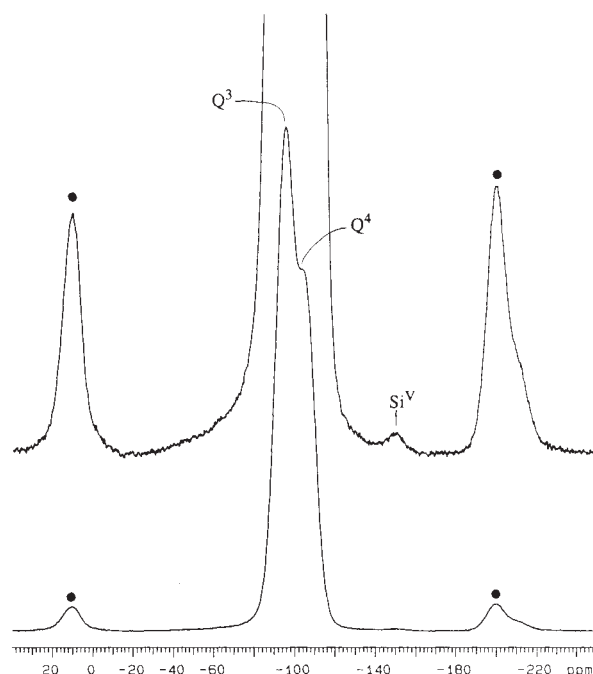


FIG. 1 The  $^{29}\text{Si}$  MAS NMR spectrum for fast-quenched  $\text{K}_2\text{Si}_4\text{O}_9$  glass. Upper trace is lower trace  $\times 10$ . Data were collected with a Varian VXR 400 spectrometer at a Larmor frequency of 79.5 MHz, using a high-speed MAS probe (Doty Scientific, Inc.) with a 5-mm rotor. A spin tip angle of  $30^\circ$  and a 1-s delay between pulses were used (using 10-s and 60-s delays produced no significant differences from the 1-s delay in the relative abundance of  $\text{Si}^{\text{V}}$  sites). Data from  $\sim 50,000$  pulses were averaged. A 20-Hz exponential line broadening was used to improve the signal-to-noise ratio. Frequency reference was tetramethyl silane. Solid circles mark spinning side bands.



of this structure<sup>9</sup> and in sodium silicophosphate glasses at 1 atmosphere pressure<sup>10</sup>.

In these high-pressure samples, peaks seen in MAS NMR spectra near to  $-150$  p.p.m. were attributed to  $\text{Si}^{\text{V}}$ , by analogy with the well-known effects of coordination number on the chemical shift of  $^{27}\text{Al}$ , by comparison with the observed shift of  $\text{Si}^{\text{V}}$  in organic molecules, and because no peaks are observed in the range from  $-125$  to  $-175$  p.p.m. for either  $\text{Si}^{\text{IV}}$  or  $\text{Si}^{\text{VI}}$  sites in crystalline silicates.

The glass described here was prepared from the sample of isotopically enriched  $\text{K}_2\text{Si}_4\text{O}_9$  (95%  $^{29}\text{Si}$ ) used in previous high-pressure and high-temperature experiments<sup>6,8,11,12</sup>. The glass was doped with 0.2 wt%  $\text{Gd}_2\text{O}_3$  to speed spin-lattice relaxation. The enrichment and the doping were essential to provide the extremely high signal-to-noise ratio needed for the observations described below. One portion of the sample was cooled slowly ( $1\text{ K min}^{-1}$  from  $1,300$  to  $300^\circ\text{C}$ ). A second portion was quenched rapidly ( $\sim 2 \times 10^4$ – $5 \times 10^5\text{ K s}^{-1}$ ) by coating a thin platinum foil with a layer of glass  $\sim 0.006\text{ mm}$  thick, heating on a gas burner, then dropping into water. After NMR data were collected, this sample was reheated to  $1,050^\circ\text{C}$  and then cooled at  $1\text{ K min}^{-1}$ .

In the frequency region for  $\text{Si}^{\text{IV}}$  ( $-60$  to  $-120$  p.p.m.), spectra are similar to those published previously (Fig. 1)<sup>6,8</sup>. Two overlapping peaks represent silicon in sites with three bridging oxygen neighbours (labelled  $\text{Q}^3$ ) and with four bridging oxygens ( $\text{Q}^4$ ). The  $\text{Si}^{\text{IV}}$  peak for the rapidly quenched glass is slightly broader (by  $0.5$  p.p.m. full width at half maximum) than that for the slow-cooled sample, as is consistent with greater disorder in the liquid at the higher fictive temperature of the former.

At  $-150$  p.p.m., a small peak is present with the same chemical shift and width ( $\sim 7$  p.p.m.) as the  $\text{Si}^{\text{V}}$  peak observed previously<sup>6,8</sup> in high-pressure samples (Figs 1 and 2). The area of this peak relative to the total peak area, which is an accurate measure of the relative abundance of the species, is  $0.06 \pm 0.02\%$  in the slow-cooled and  $0.10 \pm 0.02\%$  in the fast-cooled sample. Its area returned to  $0.06\%$  after reheating and slow cooling of the latter, indicating that the structural change is completely reversible. No peak near to  $-200$  p.p.m. was detected in spectra collected with sample spinning rate more rapid than for the data shown in the figures, indicating that the abundance of  $\text{Si}^{\text{VI}}$  was less than  $\sim 0.02\%$ .

It is at least a good first approximation that a glass records the 'frozen in' structure of a liquid at the temperature of its transition to a glass. The fictive temperatures ( $T_f$ ) of the samples described here are not precisely known, but the value should be close to  $500^\circ\text{C}$  for the slow-cooled glass<sup>13</sup>. For the fast-quenched sample,  $T_f$  can be estimated as  $665 \pm 40^\circ\text{C}$  from the activation energy for viscous flow, taken as that appropriate for structural relaxation<sup>14,15</sup>. A crude thermodynamic treatment of the observed effect of temperature on the abundance of  $\text{Si}^{\text{V}}$  can be made by calculating an equilibrium constant as the ratio of  $\text{Si}^{\text{V}}$  to  $\text{Si}^{\text{IV}}$  and applying the Van't Hoff equation to obtain an enthalpy change of  $\sim 20 \pm 10\text{ kJ mol}^{-1}$ . This result is similar to that obtained for the effect of temperature on Q species distribution<sup>15</sup>, and is of the order of the difference in the heats of formation of the cristobalite ( $\text{Si}^{\text{IV}}$ ) and stishovite ( $\text{Si}^{\text{VI}}$ ) polymorphs of  $\text{SiO}_2$  ( $47\text{ kJ mol}^{-1}$ )<sup>16</sup>. A relatively small heat effect for the Si coordination in this material is perhaps also supported by the observed small negative enthalpy of transition between crystalline  $\text{K}_2(\text{Si}^{\text{IV}})_4\text{O}_9$  and  $\text{K}_2(\text{Si}^{\text{IV}})_3(\text{Si}^{\text{VI}})\text{O}_9$  phases<sup>17</sup>.

An extrapolation of this calculation to our previous results for  $\text{Si}^{\text{V}}$  abundances (up to  $8.5\%$ ) in samples of  $\text{K}_2\text{Si}_4\text{O}_9$  and  $\text{Na}_2\text{Si}_4\text{O}_9$  glasses quenched from pressures up to  $12\text{ GPa}$  shows that at temperatures above their melting points, much or most of the silicon in these materials could have been highly coordinated. The transition to  $\text{Si}^{\text{V}}$  (and to  $\text{Si}^{\text{VI}}$ ) may thus have an even larger effect on density, on thermodynamic activities and on pressure-induced viscosity reductions than previously suspected. The latter effect has been related to correlations between

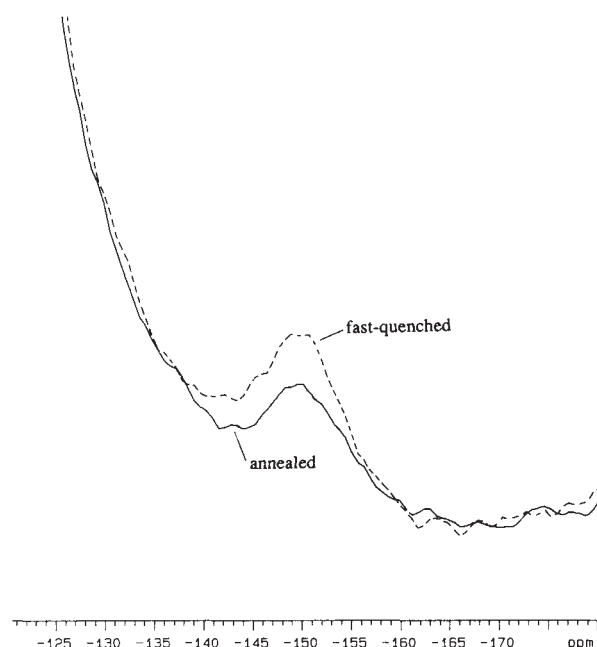


FIG. 2 Expanded  $\text{Si}^{\text{V}}$  region of spectra for fast- and slow-cooled samples. Vertical scale is 50 times that of Fig. 1. Parameters as in Fig. 1, except that  $100\text{-Hz}$  line broadening was applied.

oxygen diffusivity and the abundance of  $\text{Si}^{\text{V}}$  observed in molecular dynamics simulations of network liquids at high pressure<sup>2,5</sup>, although the simplicity of this connection has been questioned by recent molecular dynamics work on larger ensembles of particles using different potentials<sup>18</sup>.

In many liquids, mean cation coordination numbers decrease as density decreases at higher temperature, as has been observed for boron in a borosilicate liquid<sup>19</sup>. These NMR data do not reveal mean coordination number, which may still decrease with increasing temperature if other species are formed, but remain undetected spectroscopically. The increase in the abundance of  $\text{Si}^{\text{V}}$  with temperature does, however, support the idea that it plays a part in the 'weakening' of silicate liquid structure, which is expected at least in liquids with high contents of network-forming cations<sup>1,2</sup>. The results here qualitatively confirm a fundamental and general feature of theoretical models of silicate liquids. I look forward to future modelling that can be tested quantitatively against this kind of spectroscopic observation. □

Received 3 April; accepted 9 May 1991.

1. Brawer, S. *Relaxation in Viscous Liquids and Glasses* (American Ceramic Society, Columbus, Ohio, 1985).
2. Angell, C. A., Cheeseman, P. & Tamaddon, S. *Bull. Mineral.* **106**, 87–97 (1983).
3. Woodcock, L. V., Angell, C. A. & Cheeseman, P. *J. chem. Phys.* **65**, 1565–1577 (1976).
4. Soules, T. F. *J. chem. Phys.* **71**, 4570–4578 (1979).
5. Kubicki, J. D. & Lasaga, A. C. *Am. Mineral.* **73**, 941–955 (1988).
6. Stebbins, J. F. & McMillan, P. *Am. Mineral.* **74**, 965–968 (1989).
7. Xue, X., Stebbins, J. F., Kanzaki, M. & Tronnes, R. G. *Science* **245**, 962–964 (1989).
8. Xue, X., Stebbins, J. F., Kanzaki, M., McMillan, P. F. & Poe, B. *Am. Mineral.* **76**, 8–26 (1991).
9. Stebbins, J. F. & Kanzaki, M. *Science* **251**, 294–298 (1990).
10. Dupree, R., Holland, D., Mortuza, M. G., Collins, J. A. & Lockyer, M. W. G. *J. Non-Cryst. Solids* **112**, 111–119 (1989).
11. Farnan, I. & Stebbins, J. F. *J. Am. chem. Soc.* **112**, 32–39 (1990).
12. Farnan, I. & Stebbins, J. F. *J. Non-Cryst. Solids* **124**, 207–215 (1990).
13. Shelby, J. E. *J. appl. Phys.* **47**, 4489–4496 (1976).
14. Scherer, G. W. *Relaxation in Glass and Composites* (Wiley, New York, 1986).
15. Brandriss, M. E. & Stebbins, J. F. *Geochim. cosmochim. Acta* **52**, 2659–2670 (1988).
16. Robie, R. A., Hemingway, B. S. & Fisher, J. R. *US geol. Surv. Bull.* **1452** (1979).
17. Geisinger, K. L., Ross, N. L., McMillan, P. & Navrotsky, A. *Am. Mineral.* **72**, 984–994 (1987).
18. Rustad, J. R., Yuen, D. A. & Spera, F. J. *Phys. Rev. A* **42**, 2081–2089 (1990).
19. Gupta, P. K., Liu, M. L. & Bray, P. J. *J. Am. Ceram. Soc.* **68**, C-82 (1985).

ACKNOWLEDGEMENTS. This work was inspired by discussions with colleagues, particularly P. McMillan, C. A. Angell and S. Brewer, and was supported by the NSF. I thank A. Navrotsky for helpful comments.

# Biodegradation of refractory hydrocarbon biomarkers from petroleum under laboratory conditions

P. Chosson\*, C. Lanau\*, J. Connan† & D. Dessort†

\* Sanofi Elf-Biorecherches, BP 137, 31328 Labège, France

† Elf-Aquitaine, CSTJF, 64018 Pau, France

**BIOMARKERS** are of great value in petroleum exploration because they provide essential information about the geological history of oils and source rocks. Steranes<sup>1</sup> are of particular importance as they can be related to naturally occurring precursors<sup>2,3</sup>. These compounds generally experience intense biodegradation, however, which alters their original distribution<sup>4-6</sup> and obscures the information that they carry regarding oil maturity and source material. In an attempt to identify the microorganisms responsible for this degradation, we have investigated the capacity of 73 aerobic bacteria to degrade steranes present in Rozel Point (Utah) oil<sup>7</sup>. Seven Gram-positive strains, belonging to a limited number of genera, were found to be active. Using *Nocardia* sp. SEBR 16, which caused the most extensive alteration, we have determined biodegradation rates for several isomers of steranes and methylsteranes. The preference for alteration of different isomers reflects that observed in natural environments, suggesting that the degradation intermediates could be used as indicators of the extent of the biodegradation in an oil. In addition, the microorganisms used here might be effective in biodegrading oil spills.

Among the natural precursors of steranes, the most abundant and widespread are cholesterol in animals and planktonic algae,  $\beta$ -sitosterol and stigmasterol in higher plants, and ergosterol in yeasts and fungi. The tetracyclic skeleton of sterols, preserved during their conversion into hydrocarbons, is relatively resistant to the microbial attack that generally occurs when oil fields are invaded by meteoric waters. During diagenesis and catagenesis under mild thermal conditions, sterols are thus converted into steranes that retain the biological configuration  $\alpha\alpha\alpha R$  ( $5\alpha(H)$ ,  $14\alpha(H)$ ,  $17\alpha(H)$ ,  $20R$ ) of their precursors (see legend to Fig. 1 for notation). At higher temperatures an additional series of steranes, designated  $\alpha\beta\beta$  ( $5\alpha(H)$ ,  $14\beta(H)$ ,  $17\beta(H)$ ), is generated. Simultaneously, a partial side-chain isomerization occurs, leading to a mixture of the biological  $20R$  and the geological  $20S$  epimers<sup>8</sup>. These  $\alpha\alpha\alpha$  and  $\alpha\beta\beta$  steranes, which have been used for oil-to-oil<sup>9</sup> and oil-to-source-rock<sup>4</sup> correlations, are altered by bacteria, which preferentially attack the naturally occurring  $20R$  epimers. The order of preference in biodegradation is  $C_{27} > C_{28} > C_{29}$  according to the sequence  $\alpha\alpha\alpha R > \alpha\beta\beta R > \alpha\beta\beta S > \alpha\alpha\alpha S$ <sup>4-6</sup>.

To achieve sterane biodegradation under laboratory conditions, we screened pure bacterial strains inoculated on a natural sterane mixture. This strategy differed from that developed by Goodwin *et al.*<sup>5</sup>, who used whole oil and indigenous mixed population of yeasts and bacteria. We selected the Rozel Point oil from the West Rozel no. 3 well, drilled in the heavy-oil deposit of the Great Salt Lake (Utah)<sup>7</sup>. This oil was selected because of its unusual richness in regular steranes (ratio of triterpane to sterane is 0.1) which allowed us to make a gas chromatographic record of biodegradation effects easily during screening experiments. We used the branched and cyclic alkane fraction from Rozel Point oil, separated from *n*-alkanes by using a 5 Å molecular sieve<sup>10</sup>. Figure 1a shows the regular sterane pattern, analysed by gas chromatography/mass spectrometry. The mass chromatogram of  $m/z = 217$ , where  $m$  is mass (AMU) and  $z$  is charge, shows the predominance of the three biological epimers denoted 27, 28 and  $29\alpha\alpha\alpha R$ . The proportion of geological  $20S$  epimers<sup>8</sup>, assessed by the ratio of  $\alpha\alpha\alpha S$  to  $\alpha\alpha\alpha R$

and  $\alpha\beta\beta S$  to  $\alpha\alpha\alpha R$ , is routinely measured for  $C_{27}$ - and  $C_{29}$ -steranes. Low values, 0.32 and 0.27 respectively, allow us to conclude that Rozel Point oil is not very mature.

We tested 73 aerobic bacteria belonging to Pseudomonadaceae and Actinomycetaceae selected from international collections, except for *Nocardia* sp. SEBR 16, which we isolated from a soil sample. The genera examined were *Nocardia* (25 strains), *Mycobacterium* (11), *Corynebacterium* (1), *Arthrobacter* (2), *Protoaminobacter* (1), and *Pseudomonas* (33). Sterane alteration was clearly observed with seven Gram-positive species (Table 1), whereas *Pseudomonas*, Gram-negative strains, were unable to achieve such a degradation. *Nocardia* sp. SEBR 16,

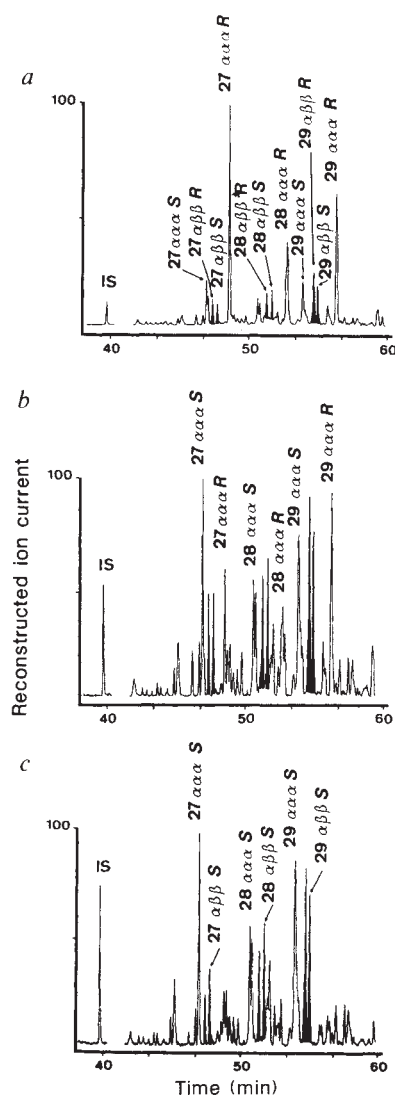


FIG. 1 Mass chromatograms of  $m/z = 217$  depicting changes in West Rozel sterane pattern using *Nocardia* sp. SEBR 16 over six days incubation. Homologous series are denoted as their atom carbon number followed by  $\alpha$  or  $\beta$  giving the position of the hydrogen atom at carbon atoms 5, 14 and 17 by referring to the plane of the sterane nucleus ( $\alpha$  indicates that H is below the plane,  $\beta$  that H is above the plane).  $R$  and  $S$  refer to the stereochemistry of the biological and geological epimers at position 20. Internal standard (IS;  $C_{26}$  *n*-alkane), added to each sample before extraction, is recorded at  $m/z = 85$ . Note the preferential removal of  $20R$  isomers (proceeding from *b* to *c*) in comparison with the blank sample incubated without bacteria (*a*). Biodegradation starts with  $C_{27}$  steranes, followed by  $C_{28}$  and  $C_{29}$ . Note also the reduction of  $\alpha\beta\beta R$ -sterane intensity (in black) relative to the internal standard. GC/MS conditions: 60-m fused capillary column coated with DB5 (0.25-mm internal diameter; 0.1- $\mu$ m film thickness); temperature programme from 40 to 130 °C at 9 °C min<sup>-1</sup>, and 130 to 300 °C at 2.5 °C min<sup>-1</sup>; carrier gas, H<sub>2</sub>. A Finnigan mat. quadrupole mass spectrometer with an INCOS data system was used.



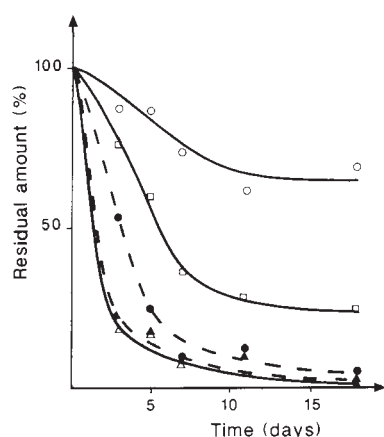


FIG. 2 Kinetics of biodegradation of pure steranes and 4 $\alpha$ -methyl steranes incubated with *Nocardia* sp. SEBR 16 over 18 days. Notation as in Fig. 1.  $\circ$ : 27 $\alpha\beta\beta$ S;  $\square$ : 27 $\alpha\beta\beta$ R;  $\bullet$ : 4 $\alpha$ -methyl-24R-ethyl- $\alpha\alpha\alpha$ R-cholestane;  $\blacktriangle$ : 4 $\alpha$ -methyl- $\alpha\alpha\alpha$ R-cholestane;  $\triangle$ : 27 $\alpha\alpha\alpha$ R. Note the rapid consumption of  $\alpha\alpha\alpha$ R components. Chromatographic conditions as in Fig. 1. Pure compounds (Chiron Laboratories, Norway), are quantified by comparison with internal standard (see Fig. 1).

which produced the most extensive biodegradation, was examined for the sequential removal of the various sterane isomers. The  $m/z = 217$  mass chromatograms (Fig. 1b and c) depict the step-by-step evolution of regular sterane distribution with an incubation time of up to six days. *Nocardia* sp. SEBR 16 degraded steranes to varying degrees with preference for biological 20R isomers. After two days of incubation (Fig. 1b),  $\alpha\alpha\alpha$ R steranes were considerably reduced, more of the  $C_{29}$  sterane remaining than of its  $C_{27}$  homologue. All of these isomers were virtually absent after six days' incubation (Fig. 1c). The more resistant  $\alpha\alpha\alpha$ S-steranes were, however, subsequently biodegraded, as measured by quantitative data (comparing their amounts to that of the internal standard). Steranes belonging to the  $\alpha\beta\beta$ R series were also preferentially altered, as shown in Fig. 1c for the  $C_{27}$  steranes. The biodegradation preference observed for both  $\alpha\alpha\alpha$  and  $\alpha\beta\beta$  steranes was  $C_{27} > C_{28} > C_{29}$ .

Methylsteranes ( $C_4$ -methyl-substituted steranes) are also present in the branched and cyclic alkane fraction from Rozel Point oil. They are important biomarkers, in particular the  $C_{30}$  methylsteranes which indicate either marine or freshwater dinoflagellate inputs<sup>11–12</sup>. In Rozel Point oil, the most abundant  $C_4$  methylsteranes are  $C_{29}$  and  $C_{30}$  compounds with the  $\alpha\alpha\alpha$ R configuration. The  $C_4$  methylsterane pattern mimics the regular sterane distribution. The evolution of  $m/z = 231$  mass chromatograms is closely similar to that observed for steranes ( $m/z = 217$ ; Fig. 1a–c), showing the preferential attack of 20R isomers.

To examine the biodegradation rates of sterane and methylsterane isomers, we used a mixture of pure compounds. We incubated  $C_{27}$  steranes of configuration  $\alpha\alpha\alpha$ R,  $\alpha\alpha\alpha$ S and  $\alpha\beta\beta$ R, and  $\alpha\alpha\alpha$ R  $C_{28}$  and  $C_{30}$  4 $\alpha$ -methylsteranes, with *Nocardia* sp. SEBR 16. The residual amount of each compound was quantified

as a function of time by comparing its area in the gas chromatogram with that of a standard added before the extraction step. The biodegradation efficiency (Fig. 2) varied according to the stereochemistry of the molecules. The  $\alpha\alpha\alpha$ R  $C_{27}$  sterane was readily and more rapidly removed than its  $\alpha\alpha\alpha$ S counterpart, an intermediate rate being observed for the  $\alpha\beta\beta$ R  $C_{27}$  sterane. All of the  $\alpha\alpha\alpha$ R 4 $\alpha$ -methylsteranes were also rapidly consumed: the  $C_{28}$  methylsterane displayed identical kinetics to those observed for the  $\alpha\alpha\alpha$ R  $C_{27}$  sterane, and the biodegradation of the  $C_{30}$  methylsterane was retarded compared with that of the  $C_{28}$  methylsterane. When considering the  $\alpha\alpha\alpha$  steranes, we observed that bacterial affinity for the 20R compound was important but unspecific, as the 20S homologue was also consumed. We can speculate that the structure of the 20R sterane may be in better concordance with the topography at the active site of the enzyme involved in the degradation. The stronger interactions between enzyme and 20R sterane could be explained by optimal distances between the sterane groups at position 20 and amino-acid residues at the active site.

We found that about 10 days were necessary for all of the components to reach their steady-state concentrations. Some of the final residual amounts were considerably greater than the insignificant values observed for the  $\alpha\alpha\alpha$ R components. We suggest that such unexpected residual amounts could be due either to the depletion of indispensable enzymatic cofactors present in the nutrient medium or to the lysis of bacterial cells.

We conclude that laboratory experiments, which lead to biomarker distributions similar to those observed in geological settings, provide reliable methods of investigating in-reservoir biodegradation of polycyclic alkanes. We can reproduce the degradation of regular steranes and their  $C_4$ -methyl substituted homologues under our laboratory conditions, the sequence being  $\alpha\alpha\alpha$ R  $>$   $\alpha\beta\beta$ R  $>$   $\alpha\beta\beta$ S  $>$   $\alpha\alpha\alpha$ S with  $C_{27} > C_{28} > C_{29}$ . Selective removal of regular steranes fully agrees with geological observations<sup>8,13–15</sup> and with laboratory experiments<sup>5</sup>. Our biodegradation assays, carried out reproducibly and more rapidly than in ref. 5, seem to be independent of the relative proportion of steranes in the oil. For instance, we see the same degree of disappearance for branched and cyclic alkanes from the terpane-rich Vic-Bilh oil (Aquitaine Basin, France) or the sterane-rich Rozel Point oil (terpane-to-sterane ratio respectively 5 and 0.1).

From these findings, some possible applications emerge. In organic geochemistry, these bacteria could be used to concentrate other bacterially resistant polycyclic biomarkers (such as hopanes and seco-hopanes) that are difficult to detect because of an excessive proportion of steranes. These bacteria may produce degradation intermediates whose characterization could allow us to establish the relationship between metabolites and the corresponding degree of sterane biodegradation. From a biological point of view, it could be interesting to investigate the metabolic pathways used by microorganisms to eliminate these molecules belonging to natural and unnatural series. Such microorganisms could help to restore environments contaminated by oil spills; they could remove tetracyclic compounds, in association with numerous banal bacteria known to be efficient consumers of linear hydrocarbons. Finally, we have confirmed that sterane-degrading bacteria belong to the genera reported to transform a wide range of sterols and steroids. We think that a similar experimental approach could determine the microorganisms that alter the more resistant terpane biomarkers.  $\square$

TABLE 1 Gram-positive bacteria active for the degradation of steranes in branched and cyclic alkane fraction from Rozel Point oil

| Strain                       | ATCC*  |
|------------------------------|--------|
| <i>Arthrobacter simplex</i>  | 13,260 |
| <i>Mycobacterium</i> sp.     | 29,472 |
| <i>Nocardia erythropolis</i> | 4,277  |
| <i>Nocardia globerulea</i>   | 21,505 |
| <i>Nocardia globerulea</i>   | 21,506 |
| <i>Nocardia</i> sp.          | 19,170 |
| <i>Nocardia</i> sp. SEBR 16  | —      |

\* American Type Culture Collection, Rockville, Maryland, USA.

Received 16 November 1990; accepted 29 April 1991.

- Mackenzie, A. S. in *Advances in Petroleum Organic Geochemistry* (eds Brooks, J. & Welte, D. H.) 115–124 (Academic, London, 1984).
- Dastillung, M. & Albrecht, P. *Nature* **269**, 678–679 (1977).
- Ensminger, A., Joly, G. & Albrecht, P. *Tetrahedron Lett.* 1575–1578 (1978).
- Seifert, W. K. & Moldovan, J. M. *Geochim. cosmochim. Acta* **42**, 77–95 (1978).
- Goodwin, N. S., Park, J. D. & Rawlinson, A. P. in *Advances in Organic Geochemistry* (eds Bjorøy, M. et al.) 650–658 (Wiley, Chichester, 1983).

6. McKirdy, D. M., Aldridge, A. K. & Ypma, P. J. M. in *Advances in Organic Geochemistry* (eds Bjorøy, M. et al.) 99–107 (Wiley, Chichester, 1983).
7. Bortz, L. C. in *Exploration for Heavy Crude Oil and Bitumen* (ed. Meyer, R. R.) 555–563 (AAPG Studies in Geology, Tulsa, USA, 1987).
8. Seifert, W. K. & Moldovan, J. M. *Geochim. cosmochim. Acta* **43**, 111–126 (1979).
9. Siefert, W. K., Moldovan, J. M. & Demaison, G. J. *Org. Geochem.* **6**, 633–643 (1984).
10. O'Connor, J. G., Burrow, F. H. & Norris, M. S. *Analyt. Chem.* **34**, 82–85 (1962).
11. Goodwin, N. S., Mann, A. L. & Patience, R. L. *Org. Geochem.* **12**, 495–506 (1988).
12. Summons, R. E., Volkman, J. K. & Boreham, C. J. *Geochim. cosmochim. Acta* **51**, 3075–3082 (1987).
13. Rullkötter, J. & Wendisch, D. *Geochim. cosmochim. Acta* **46**, 1545–1552 (1982).
14. Volkman, J. K., Alexander, R., Kagi, R. I. & Woodhouse, G. W. *Geochim. cosmochim. Acta* **47**, 785–794 (1983).
15. Landais, P. & Connan, J. *Sci. Géol. Bull.* **39**, 293–314 (1986).

ACKNOWLEDGEMENTS. We thank F. Bernard for his continuous support and the Société Nationale Elf-Aquitaine for its financial contribution.

## Enrichment in saturated compounds of Black Sea interfacial sediment

J. A. Beier<sup>\*§</sup>, Stuart G. Wakeham<sup>\*</sup>, C. H. Pilskaln<sup>†</sup> & S. Honjo<sup>‡</sup>

<sup>\*</sup>Skidaway Institute of Oceanography, Box 13687, Savannah, Georgia 31416, USA

<sup>†</sup>Monterey Bay Aquarium Research Institute, Monterey, California 93950, USA

<sup>‡</sup>Department of Geology and Geophysics, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543, USA

THE sediment/water interface of the ocean should consist of organic compounds produced both in the overlying water column and during early diagenesis<sup>1–5</sup>. We have found that the uppermost ~1 mm of sediment samples obtained from the Black Sea are enriched in saturated sterols and fatty acids relative to both suspended particles and underlying sediments. We speculate that either this 'floc' layer contains a microbial community capable of yielding a distribution of compounds that is high in saturated species, or the floc accumulates low-density, fine material enriched in saturated components produced in the water column<sup>6</sup>. Saturated components would thus be formed or concentrated very early during sedimentation and diagenesis. This has important implications for hydrogenation rates in geological environments, and may cast light on the way in which biological materials, rich in unsaturated compounds, become converted into sediments, petroleum and source rocks that contain predominantly saturated compounds.

The upper sediments in the Black Sea ('Unit 1') consist of alternating coccolith-rich carbonate/organic-rich varve couplets. Box cores from the 1988 Black Sea expedition showed the varved sequence to be overlain by 2–4 cm of unconsolidated sediment referred to as 'fluff'<sup>7</sup>. The fluff is of sufficiently low density that zooplankton faecal pellets sink through it and are preserved at the fluff/Unit 1 boundary<sup>8</sup>. We sampled the upper ~1 mm of flocculant material at the surface of the fluff (using a large syringe in an undisturbed box core) to assess the transfer of organic material from the water column into the sediment. This 'floc' was an organic-rich coccolith-ooze containing numerous spicules of *Rizosolenia* sp., an important Black Sea diatom. The floc and underlying 0–1-cm interval of sediment from three stations (BS5-BC10 (Station BSK-2; 43° 05' N, 34° 00' E, water depth 2,200 m); BS5-BC16 (41° 51' N, 28° 50' E, 600 m); and BS5-BC1 (41° 38' N, 28° 59' E, 170 m)) were analysed for lipids (see ref. 9 for analytical details). For comparison, faecal pellets from the fluff/Unit 1 boundary and sediment-trap material from a 3-month deployment at 400 m in the anoxic zone at BSK-2 were also analysed. We anticipated that the composition of the floc would be intermediate between water-

column particles and the underlying sediment; but on the contrary, it has an unusual composition that is distinct from either of these.

Sterol and carboxylic (fatty) acid distributions (Figs 1 and 2 for BS5-BC10) illustrate the composition of the floc layer. Sterol and acid concentrations (27 and 36 µg per g dry weight, respec-

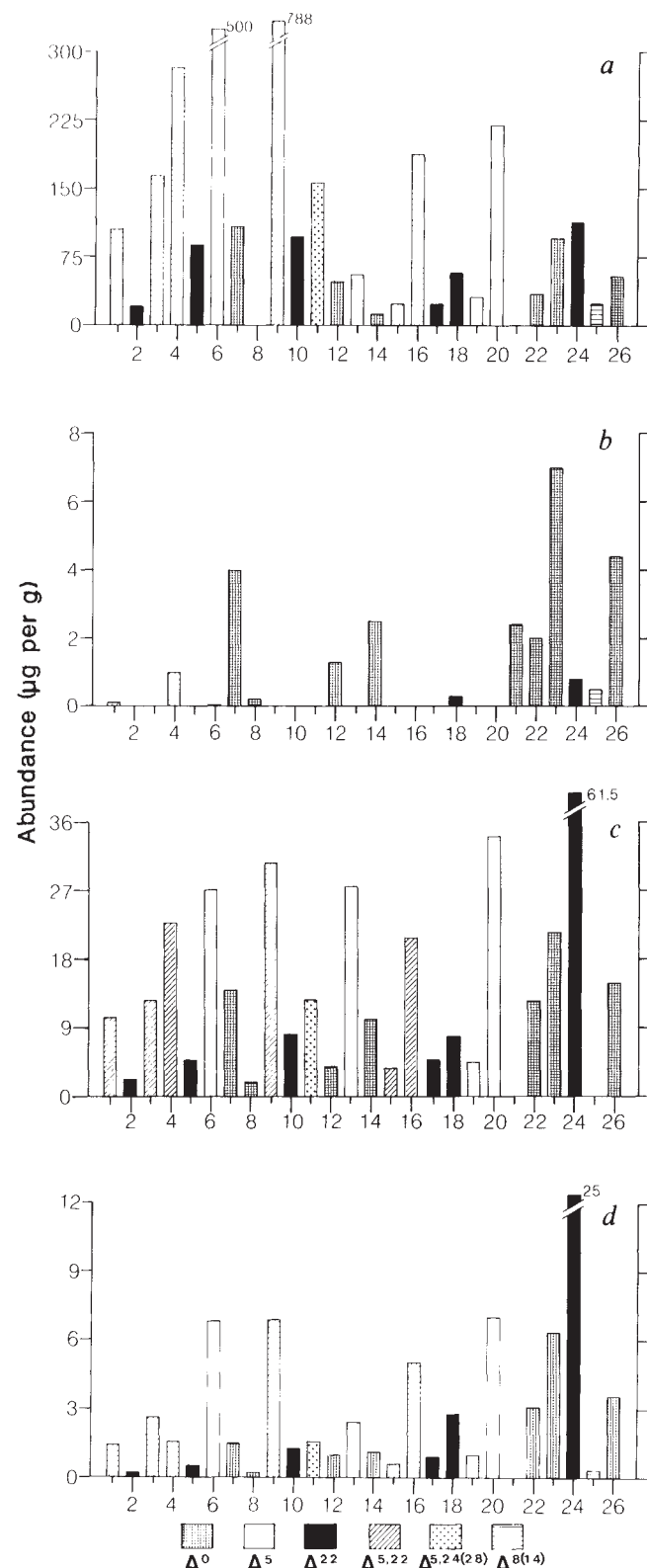


FIG. 1 Distribution and abundance of sterols in Black Sea samples from station BSK-2. a, Sediment trap (400 m); b, floc; c, 0–1 cm interval of fluff; d, faecal pellets. Bar numbers on histograms refer to structural assignments of sterols listed in Table 2. The Δ notation indicates the double bond position.

§ Present address: ST Systems Corporation, 7601 Ora Glen Drive, Suite 300, Greenbelt, Maryland 20770, USA.



tively) were markedly lower than in the underlying 0–1-cm fluff sediment (370 and 142  $\mu\text{g}$  per g, respectively) and the sediment trap material (3,300 and 3,600  $\mu\text{g}$  per g). The concentration differences between the floc and the fluff were all the more unusual because they have similar total organic carbon contents, carbon isotopic compositions and calcium/aluminium ratios (Table 1). The fluff had a composition similar to sediments throughout the 20-cm core, although there was a decrease in lipid concentration with depth.

The key compositional feature of the floc was the predominance of saturated compounds, yielding a marked decoupling of the water-column particles, floc and underlying sediment. The dominant sterols in the floc were fully saturated stanols (ring and side-chain saturated; sterols 7, 14, 23, and 26 in Fig. 1). Unsaturated sterols made up only 4% of total sterols in the floc but 64% in the fluff (Table 1). Similarly, the floc was rich in saturated fatty acids (dominant monocarboxylic acids are 5, 7, and 10 in Fig. 2); unsaturated fatty acids made up 4% of the total fatty acid content (Table 1).

In contrast, sterol and fatty acid distributions in sediment-trap particles, fluff material from 0–1 cm and faecal pellets contained abundant unsaturated compounds typical of plankton and terrestrial material. Sterols 4, 6, 9 and 24 in Fig. 1 are common sterols in diatoms, prymnesiophytes and dinoflagellates, all principal phytoplankton in the Black Sea<sup>10</sup>. Sterols 13 and 20 may be derived from terrestrial sources. Among the fatty acids, chief planktonic constituents in the sediment-trap sample included  $\text{C}_{14:0}$  and  $\text{C}_{16:0}$  (where the number after the colon indicates the number of double bonds: these are saturated acids) and unsaturated  $\text{C}_{16}$ ,  $\text{C}_{18}$ ,  $\text{C}_{20}$  and  $\text{C}_{22}$  acids (9, 16, 19 and 21 in Fig. 2). Metabolism of fatty acids by zooplankton produces faecal pellets depleted in unsaturated fatty acids, and typically enriched in zooplankton-derived 18:1 $\omega$ 9 ( $\omega$  indicates position of double bond) and 20:0 components (16 and 18 in Fig. 2). The 0–1-cm sediment sample contained 16:1 $\omega$ 7 (9) and iso- and anteiso-pentadecanoic acids (6 and 7) attributable to *in situ* anaerobic bacterial activity in the water column and sediment<sup>9</sup>.

The delivery of material to the sediment–water interface is dominated by a few rapidly sinking large particles; fine particles are more abundant but sink slowly and contribute little to the flux<sup>11</sup>. Large particles include macroaggregates and faecal pellets, presumably the type of material collected in the sediment trap. Dense, coccolith-packed pellets and fragments can sink through the sedimentary fluff layer to be concentrated near the top of the varved sediment<sup>8</sup>. The organic matter in the pellets thus may contribute little to the floc or fluff, but rather more to the semi-consolidated sediment below. These pellets could also bypass any microbial processes taking place specifically in the water column.

As the floc layer is representative of neither water-column particles nor the underlying sediment, what processes might

yield such an organic composition? The floc may result from intense *in situ* anaerobic alteration at the sediment–water interface. Preferential removal of unsaturated lipids, either by preferential loss of unsaturated components relative to saturated compounds or by conversion of unsaturated to saturated compounds through hydrogenation of double bonds, occurs during early

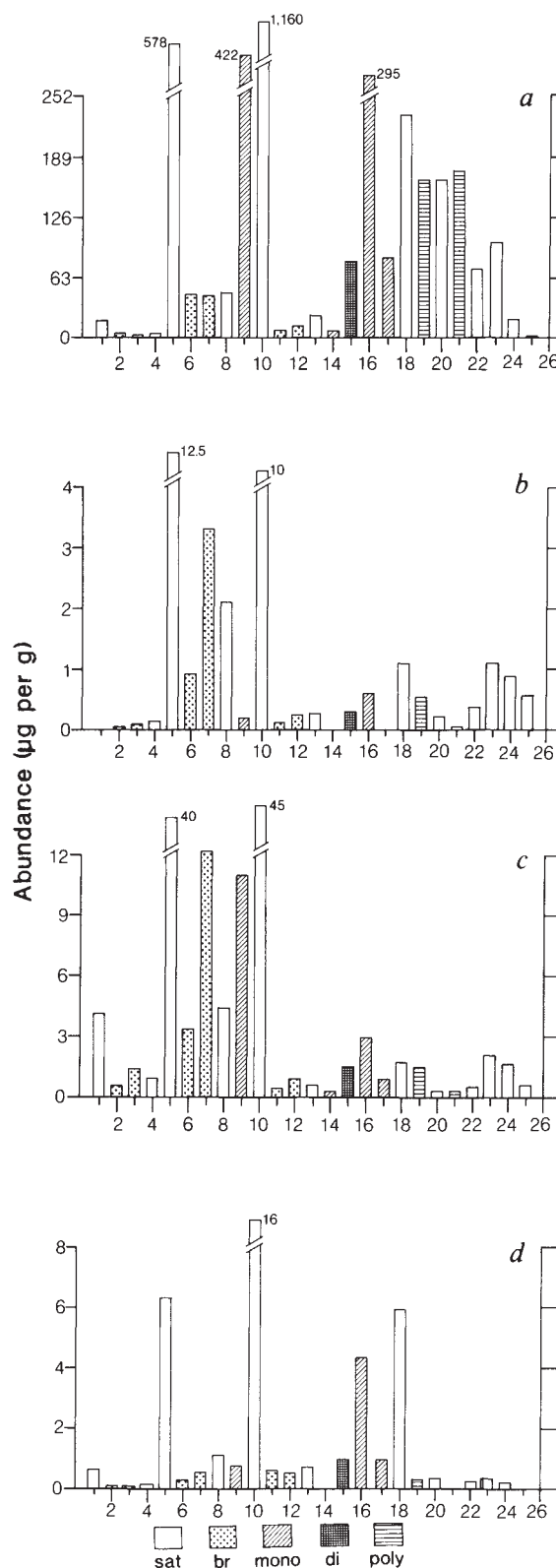


FIG. 2 Distribution and abundance of monocarboxylic fatty acids in Black Sea samples from station BSK-2. a, Sediment trap; b, floc; c, 0–1-cm interval of fluff; d, faecal pellets. Bar numbers refer to structural assignments of sterols listed in Table 2. In the key, br stands for branched.

TABLE 1 Geochemistry of Black Sea samples from station BSK-2

|                                                            | Floc  | 0–1-cm<br>fluff | Faecal<br>pellets | Sediment<br>trap |
|------------------------------------------------------------|-------|-----------------|-------------------|------------------|
| Total organic carbon (wt%)                                 | 3.2   | 3.2             | —                 | —                |
| C/N (atomic)                                               | 11    | 14              | —                 | —                |
| Ca/Al                                                      | 2.4   | 2.6             | —                 | —                |
| $\delta^{13}\text{C}_{\text{org}}$ (‰)*                    | –24.0 | –24.5           | —                 | —                |
| $\delta^{13}\text{C}_{\text{carb}}$ (‰)*                   | +3.7  | +3.9            | —                 | —                |
| Total sterols<br>( $\mu\text{g}$ per g dry weight)         | 28    | 325             | 85                | 3,300            |
| $\Delta^{\circ}\text{stanol}/\Delta^{\circ}\text{-stenol}$ | 182   | 0.4             | 0.33              | 0.2              |
| Total fatty acids†<br>( $\mu\text{g}$ per g dry weight)    | 33    | 137             | 41                | 3,650            |
| Sat./unsat. fatty acids‡                                   | 18.1  | 6.2             | 4.7               | 2.0              |

\*  $\delta^{13}\text{C} = [({}^{13}\text{C}/{}^{12}\text{C})_{\text{sample}}/({}^{13}\text{C}/{}^{12}\text{C})_{\text{PDB}}] - 1$ .

† Total  $\text{C}_{14}$ – $\text{C}_{26}$  monocarboxylic acids.

‡  $\text{C}_{14}$ – $\text{C}_{22}$  fatty acids.

diagenesis<sup>13,14</sup>, although typically not to the degree observed here. Increasing ratios of stanol to stanol reflect these processes, but such ratios are rarely much greater than 1; a 5 $\alpha$ (H)-cholestan-3 $\beta$ -ol/cholest-5-en-3 $\beta$ -ol ratio of 0.5 for the 0–1-cm fluff is typical for Black Sea sediments<sup>9,14</sup>. Remarkably, the ratio for the floc was 133.

Although stanols could be actively reduced to stanols in the floc, the stanols could be already predominant in the sedimenting matter, or be preserved preferentially in the floc. Stanols are abundant in dinoflagellate-derived organic matter (especially 4-methyl stanols)<sup>16–19</sup>, but so are polyenoic C<sub>18</sub>–, C<sub>20</sub>–, and C<sub>22</sub>-fatty acids, which are not abundant in the floc. And studies of stanol–stanol transformations<sup>13,14</sup> do not indicate selective preservation of either stanols or stanols under anoxic conditions. A preferential loss of unsaturated fatty acids and subsequent enrichment of saturated components is the typical early diagenetic fate of fatty acids<sup>12,15</sup>, but the considerable shift in composition seen here has not been observed elsewhere. If the unusual organic composition of the floc results from intense *in situ* microbial reduction at the sediment–water interface, then these processes may be especially important in euxinic settings.

Fine (< 53  $\mu$ m) particles, which might preferentially accumulate in the floc<sup>20</sup>, are depleted in unsaturated fatty acids and sterols relative to large (> 53  $\mu$ m) particles in the Black Sea<sup>6</sup>. This suggests that fine material, with slow settling rates and enhanced loss of unsaturated components, could be a source of some of the saturated lipids in the floc. But fine particles in the water column were still not as enriched in saturated components as was the floc.

The floc layer may eventually be buried in the sediment column, and if such layers have formed previously at the sediment–water interface, then the sediment record could contain many buried floc layers. Alternatively, the floc may constitute a 'living mat' which remains at the interface and is seldom buried. But the floc layer is thin (a fraction of a centimetre), and the concentrations of sterols and fatty acids in the floc (Table 1) are markedly lower than in the fluff. Therefore, the

predominance of saturated lipids might not be detected in sediment–core samples even if they did contain buried floc layers.

The observation of interfacial flocs having unique organic compositions at three locations (one in the central basin, two in the margin) in the Black Sea indicates that the processes involved are not restricted to the deep central basin. Oxygen depletion of the bottom waters probably has an important role, in that euxinic environments may contain microbial communities efficient in reducing and/or removing unsaturated compounds, and bioturbation or physical mixing processes may prevent the build-up of a flocculent layer having a composition such as that in the Black Sea. We do not know whether this floc is unique to the Black Sea and its anoxic bottom waters. It may be that similar interfacial sediment exists elsewhere, and that our sampling strategy in the Black Sea led to this serendipitous discovery. □

Received 5 November 1990; accepted 19 April 1991.

1. Burns, K. A. *Reun. Cons. Int. Explor. Mer* **186**, 432–441 (1986).
2. Smith, D. J., Eglinton, G. & Morris, R. J. *Nature* **304**, 259–262 (1982).
3. Smith, D. J., Eglinton, G., Morris, R. J. & Poutanen, E. *Oceanologica Acta* **6**, 211–219 (1983).
4. Wakeham, S. G. & Lee, C. *Org. Geochem.* **14**, 83–96 (1989).
5. Lee, C., Farrington, J. W. & Gagosian, R. B. *Geochim. cosmochim. Acta* **43**, 35–46 (1979).
6. Wakeham, S. G. *Nature* **342**, 787–790 (1989).
7. Honjo, S. *et al. Woods Hole Oceanogr. Inst. Tech. Rep. No.* 88–35 (1988).
8. Pilska, C. *EOS* **69**, 1243 (1989).
9. Wakeham, S. G. & Beier, J. A. *Deep Sea Res.* (in the press).
10. Benli, H. A. in *SCOPE/UNEP Sonderband Heft 62 Mitt. geol. paläontol. Inst. Univ. Hamburg* 77–87 (1979).
11. McCave, I. N. *Deep Sea Res.* **22**, 491–502 (1979).
12. Kawamura, K. & Ishiwatari, R. *Geochim. cosmochim. Acta* **42**, 349–357 (1978).
13. Nishimura, M. *Geochim. cosmochim. Acta* **48**, 251–266 (1984).
14. Gagosian, R. B., Smith, S. O., Lee, C., Farrington, J. W. & Frew, N. M. in *Advances in Organic Geochemistry 1979* (eds Douglas, A. G. & Maxwell, J. R.) 407–419 (1980).
15. VanVleet, E. S. & Quinn, J. G. *Geochim. cosmochim. Acta* **43**, 289–303 (1979).
16. Nichols, P. D., Jones, G. J., DeLeeuw, J. W. & Johns, R. B. *Phytochemistry* **23**, 1043–1047 (1984).
17. Robinson, N., Cranwell, P. A., Eglinton, G. & Jaworski, G. H. M. *Phytochemistry* **26**, 411–421 (1987).
18. Harvey, J. R., Bradshaw, S. A., O'Hara, S. C. M., Eglinton, G. & Corner, E. D. S. *Phytochemistry* **27**, 1723–1729 (1988).
19. Volkman, J. K., Gagosian, R. B. & Wakeham, S. G. *Lipids* **19**, 457–462 (1984).
20. Pilska, C. H. in *Woods Hole Oceanogr. Inst. Tech. Rep. No.* 88–35 (eds Honjo, S. & Hay, B.) 141–143 (1988).

ACKNOWLEDGEMENTS. We thank C. H. Clifford for aid in sampling, P. Whaling, R. Smith and A. J. Kaufman for assisting in geochemical analysis and A. Boyette for drafting the figures. Funding was provided by ONR (S.G.W.) and NSF (S.H. and C.H.P.). Ship support for the Black Sea expedition was funded by NSF.

TABLE 2 Fatty acids and sterols in Black Sea samples

| Designation | Fatty acid assignment (Fig. 1) | Sterol assignment (Fig. 2)                                              |
|-------------|--------------------------------|-------------------------------------------------------------------------|
| 1           | n-12:0                         | 24-norcholesta-5,22E-dien-3 $\beta$ -ol                                 |
| 2           | iso-13:0                       | 27-nor-5 $\alpha$ (H)-cholest-22E-en-3 $\beta$ -ol                      |
| 3           | anteiso-13:0                   | 27-nor-24-methylcholesta-5,22E-dien-3 $\beta$ -ol                       |
| 4           | n-13:0                         | cholesta-5,22E-dien-3 $\beta$ -ol                                       |
| 5           | n-14:0                         | 5 $\alpha$ (H)-cholest-22E-en-3 $\beta$ -ol                             |
| 6           | iso-15:0                       | cholest-5-en-3 $\beta$ -ol                                              |
| 7           | anteiso-15:0                   | 5 $\alpha$ (H)-cholestan-3 $\beta$ -ol                                  |
| 8           | n-15:0                         | 27-nor-24-methyl-5 $\alpha$ (H)-cholestan-3 $\beta$ -ol                 |
| 9           | 16:1                           | 24-methylcholesta-5,22E-dien-3 $\beta$ -ol                              |
| 10          | n-16:0                         | 24-methyl-5 $\alpha$ (H)-cholest-22E-en-3 $\beta$ -ol                   |
| 11          | iso-17:0                       | 24-methylcholesta-5,24(28)-dien-3 $\beta$ -ol                           |
| 12          | anteiso-17:0                   | 4-methyl-5 $\alpha$ (H)-cholestan-3 $\beta$ -ol                         |
| 13          | n-17:0                         | 24-methylcholest-5-en-3 $\beta$ -ol                                     |
| 14          | 17:1                           | 24-methyl-5 $\alpha$ (H)-cholestan-3 $\beta$ -ol                        |
| 15          | 18:2                           | 23,24-dimethylcholesta-5,22E-dien-3 $\beta$ -ol                         |
| 16          | 18:1 $\Delta^9$                | 24-ethylcholesta-5,22E-dien-3 $\beta$ -ol                               |
| 17          | 18:1 $\Delta^{11}$             | 24-ethyl-5 $\alpha$ (H)-cholest-22E-en-3 $\beta$ -ol                    |
| 18          | n-18:0                         | 4,24-dimethyl-5 $\alpha$ (H)-cholest-22E-en-3 $\beta$ -ol               |
| 19          | 20:5                           | 23,24-dimethylcholest-5-en-3 $\beta$ -ol                                |
| 20          | n-20:0                         | 24-ethylcholest-5-en-3 $\beta$ -ol                                      |
| 21          | 22:6                           | 23,24-dimethyl-5 $\alpha$ (H)-cholestan-3 $\beta$ -ol                   |
| 22          | n-22:0                         | 24-ethyl-5 $\alpha$ (H)-cholestan-3 $\beta$ -ol                         |
| 23          | n-24:0                         | 4 $\alpha$ ,24-dimethyl-5 $\alpha$ (H)-cholestan-3 $\beta$ -ol          |
| 24          | n-26:0                         | 4 $\alpha$ ,23,24-trimethyl-5 $\alpha$ (H)-cholest-22E-en-3 $\beta$ -ol |
| 25          | n-28:0                         | 4 $\alpha$ ,23,24-trimethylcholest-8(14)-en-3 $\beta$ -ol               |
| 26          |                                | 4 $\alpha$ ,23,24-trimethyl-5 $\alpha$ (H)-cholestan-3 $\beta$ -ol      |

## Rapid change in strontium isotopic composition of sea water before the Cretaceous/Tertiary boundary

Bruce K. Nelson, G. Kenneth MacLeod & Peter D. Ward

Department of Geological Sciences, AJ-20, University of Washington, Seattle, Washington, USA

A CRITICAL aspect of the debate over the origin of the chemical, biological and climatological perturbations that characterize the Cretaceous/Tertiary boundary (K/T boundary) is the timescale and detailed pattern of the extinctions and geochemical changes. Whether the development of the anomalies was catastrophic (over tens to hundreds of years) or more gradual ( $10^3$  to  $10^6$  years), whether the anomalies occur exactly at the boundary or precede it, and whether the chemical and biological signatures represent one event or can be resolved into several discrete events, are issues fundamental to identifying the cause of the marked changes. To determine the timing and rate of change of strontium isotope evolution of sea water during the five million years preceding the K/T boundary, we analysed foraminifera from an exceptionally thick, palaeontologically well-characterized marine K/T section



exposed at Bidart, southwest France. We found a rapid increase in  $^{87}\text{Sr}/^{86}\text{Sr}$  of ocean water 1.5 to 2.3 Myr before the boundary. The increase may be explained by a 10% increase in continental strontium flux to the oceans over a period of about 1 Myr.

Compilations of previous studies of the evolution of the  $^{87}\text{Sr}/^{86}\text{Sr}$  ratio in sea water<sup>1-4</sup> by Macdougall<sup>5</sup> and by Javoy and Courtillot<sup>6</sup> emphasized a distinct positive spike of  $1-2 \times 10^{-4}$  above the ambient isotope ratio before and after the K/T boundary. Macdougall inferred a sharp rise in the ratio exactly at the K/T boundary, supporting the idea of a catastrophic meteorite impact. In contrast, using the same data set, Javoy and Courtillot inferred an increase in the ratio during the 2-3 Myr before the K/T boundary, in support of large-scale volcanism. Most isotope data were obtained from Deep-Sea Drilling Project (DSDP) drill cores<sup>1-3</sup>, for which samples were assigned ages with errors of  $\pm 2-3$  Myr. In the most intensely studied core spanning the 2-3 Myr before the K/T boundary<sup>1</sup>, only three samples distributed over 28 m of section were analysed. Uncertainties in both the age assignments for individual samples and in correlations among different cores, as well as the small number of analysed samples, make it impossible to infer the timing and shape of the strontium isotope anomaly unambiguously.

To reduce these uncertainties, we analysed 19 samples from a single section that spans most of Maastrichtian time. The Maastrichtian section at Bidart, near Biarritz, southwest France, is at least 180-m thick, and is composed of limestone and marls deposited in the Basque-Cantabric Basin. All standard planktonic foraminifera and calcareous nannoplankton zones are present, suggesting a relatively complete section<sup>7,8</sup> (Fig. 1). The first appearance of *A. mayaroensis*, 2.5 Myr before the K/T boundary<sup>9</sup>, is 108 m below the K/T boundary, near the base of member III. This indicates an average, compacted sedimentation rate of 40 mm kyr<sup>-1</sup>.

Well-preserved foraminifera fragments were picked from disaggregated and washed bulk samples. The samples were cleaned by ultrasonic treatments in water, further handpicking and dissolution of the sample carbonate in 5M acetic acid to minimize

contamination from non-carbonate material<sup>10</sup>. Samples dissolved in 0.3M HCl gave the same values as the material soluble in acetic acid. Measured ratios of  $^{87}\text{Rb}/^{86}\text{Sr}$  were less than 0.001, requiring insignificant corrections for *in situ* radioactive decay. We estimate the accuracy and precision of the isotope analyses at  $\pm 0.000026$  ( $2\sigma$ ) based on 20 analyses of NBS 987 during the period of investigation. Ninety per cent of the replicate sample analyses agree to within 26 p.p.m. of the sample average (Table 1).

The variation of measured  $^{87}\text{Sr}/^{86}\text{Sr}$  ratios with stratigraphic position (Fig. 2) unambiguously shows that the isotope anomaly is first resolvable at the beginning of the Late Maastrichtian,  $\sim 90$  m below the K/T boundary, within the *L. praequadratus* nannofossil zone<sup>8</sup>. The isotopic ratio reaches a maximum value 40-50 m below the K/T boundary, in the upper *L. quadratus*

TABLE 1 Isotopic composition of strontium in Bidart foraminifera

| Sample   | Distance below K/T boundary (m) | $^{87}\text{Sr}/^{86}\text{Sr}^*$ | $\delta^{87}\text{Sr}^\dagger$ | Average $^{87}\text{Sr}/^{86}\text{Sr}$ |
|----------|---------------------------------|-----------------------------------|--------------------------------|-----------------------------------------|
| B-74831  | -0.05                           | 0.707837                          | -187                           | 0.707837                                |
| B-74830  | 0.05                            | 0.707837                          | -187                           |                                         |
|          |                                 | 0.707810                          | -191                           | 0.707818                                |
|          |                                 | 0.707805                          | -191                           |                                         |
|          |                                 | 0.707824                          | -189                           |                                         |
|          |                                 | 0.707832                          | -187                           |                                         |
| B-74827  | 15                              | 0.707802                          | -192                           | 0.707793                                |
|          |                                 | 0.707784                          | -194                           |                                         |
| B-74826  | 20                              | 0.707781                          | -195                           | 0.707781                                |
| B-74825  | 40                              | 0.707786                          | -194                           |                                         |
|          |                                 | 0.707831                          | -188                           | 0.707815                                |
|          |                                 | 0.707809                          | -191                           |                                         |
|          |                                 | 0.707834                          | -187                           |                                         |
|          |                                 | 0.707799                          | -192                           |                                         |
| B-74707  | 45                              | 0.707834                          | -187                           | 0.707818                                |
|          |                                 | 0.707808                          | -191                           |                                         |
|          |                                 | 0.707829                          | -188                           | 0.707801                                |
| B-74823  | 50                              | 0.707784                          | -194                           |                                         |
|          |                                 | 0.707821                          | -189                           | 0.707780                                |
|          |                                 | 0.707799                          | -192                           |                                         |
| B-74821  | 60                              | 0.707780                          | -195                           | 0.707748                                |
| B-74819  | 68                              | 0.707780                          | -195                           |                                         |
|          |                                 | 0.707722                          | -203                           | 0.707750                                |
|          |                                 | 0.707755                          | -198                           |                                         |
|          |                                 | 0.707768                          | -197                           | 0.707746                                |
| B-74818  | 80                              | 0.707764                          | -197                           |                                         |
|          |                                 | 0.707742                          | -200                           | 0.707743                                |
| B-74817  | 85                              | 0.707746                          | -200                           |                                         |
|          |                                 | 0.707743                          | -200                           | 0.707718                                |
|          |                                 | 0.707730                          | -202                           |                                         |
| B-74816  | 90                              | 0.707765                          | -197                           | 0.707714                                |
|          |                                 | 0.707747                          | -199                           |                                         |
| B-74813‡ | 100                             | 0.707740                          | -200                           | 0.707704                                |
| B-74813  | 110                             | 0.707720                          | -203                           |                                         |
| B-74812‡ | 110                             | 0.707716                          | -204                           | 0.707719                                |
| B-74811  | 120                             | 0.707714                          | -204                           |                                         |
|          |                                 | 0.707695                          | -207                           | 0.707706                                |
|          |                                 | 0.707713                          | -204                           |                                         |
| B-74809  | 130                             | 0.707728                          | -202                           | 0.707702                                |
|          |                                 | 0.707711                          | -205                           |                                         |
| B-74806  | 145                             | 0.707707                          | -205                           | 0.707686                                |
| B-74803  | 155                             | 0.707706                          | -205                           |                                         |
|          |                                 | 0.707705                          | -205                           | 0.709161                                |
| B-74800  | 170                             | 0.707699                          | -206                           |                                         |
|          |                                 | 0.707689                          | -208                           | 0.709160                                |
|          |                                 | 0.707684                          | -208                           |                                         |
| Seawater |                                 | 0.709163                          | 0.2                            |                                         |
|          |                                 | 0.709160                          | -0.2                           |                                         |

\* Isotopic ratios are normalized to NBS 987 = 0.71024. Mass discrimination corrected to  $^{86}\text{Sr}/^{86}\text{Sr} = 0.1194$ . Errors are  $\pm 26 \times 10^{-6}$ .

†  $\delta^{87}\text{Sr} = \{^{87}\text{Sr}/^{86}\text{Sr}_{\text{sample}} / ^{87}\text{Sr}/^{86}\text{Sr}_{\text{seawater}} - 1\} \times 10^5$ .

‡ Sample treated by 0.3M HCl; all other samples treated by 5M acetic acid.

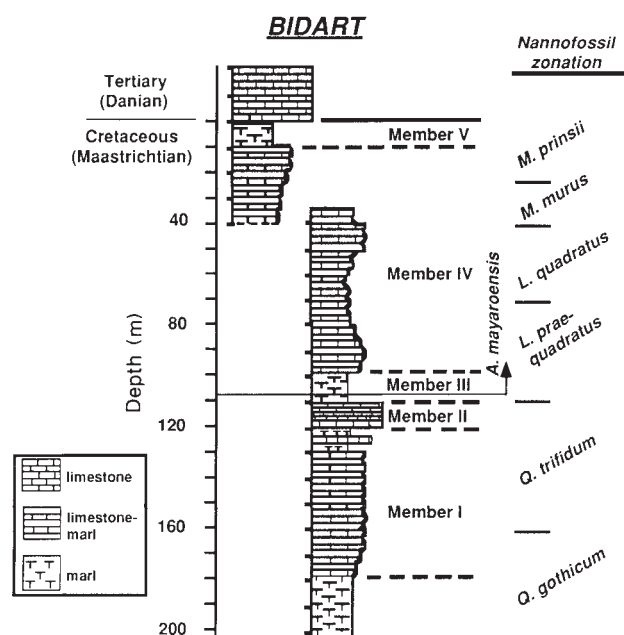


FIG. 1 Stratigraphic section showing lithologic units and nannofossil zones of sampled locality. The two parts of the section are separated by a fault. The uncertainty in relative depth below the K/T boundary introduced by correlation of the two parts of the section is  $\sim 10$  m. Nannofossil zonation is from Clausen<sup>8</sup>.

zone. From this time until the earliest Danian (through the *M. murus* zone) the  $^{87}\text{Sr}/^{86}\text{Sr}$  ratio fluctuates only slightly. Although not strictly resolvable at the 95% confidence level, a small isotope spike of  $0.2 \times 10^{-4}$  may exist at the K/T boundary; this possible boundary spike is not, however, the  $1 \times 10^{-4}$  anomaly identified by previous workers. Before the increase, the isotope ratios in samples back into the Early Maastrichtian seem to decrease gradually. The increase in the  $^{87}\text{Sr}/^{86}\text{Sr}$  ratio in sea water from Early to Late Maastrichtian is  $1.2 \times 10^{-4}$ , in agreement with the magnitude of the spike ( $1 \times 10^{-4}$ ) identified by Hess *et al.*<sup>1</sup>. When normalized to modern sea water (Table 1), the measured isotopic composition of the most radiogenic samples are within the error of previous analyses<sup>1-3</sup>.

The change in isotopic composition of the oceans did not coincide with the main foraminiferal extinctions that define the K/T boundary, but it did roughly coincide with a global decline of abundance and diversity in inoceramid bivalves<sup>11</sup>, and possibly in rudist bivalves (ref. 12, and N. H. M. Swinburne, personal communication). The onset of the rapid isotopic shift, near the base of Unit III, coincides with the last observed inoceramid body fossil at Bidart<sup>11</sup>.

Neither bioturbation nor diagenesis seems to have significantly altered the original sea-water strontium isotope signal or the shape of the anomaly in the Bidart section. Bioturbation typically vertically mixes up to  $\sim 10$  cm of uncompacted ( $\sim 5$  cm of compacted) sediment<sup>13</sup>. The sampling interval for this study is two to three orders of magnitude greater than the interval mixed by bioturbation, and the isotope shift spans at least seven samples. Modification of the original isotopic composition of foraminifera is also possible by recrystallization and exchange with pore fluids during burial, or by interaction with ground-water moving along fractures much later during uplift and exposure. Incorporation of strontium from pore waters during recrystallization of carbonate after deposition probably had little effect on the measured isotope pattern. From study of DSDP core material, Richter and DePaolo<sup>14,15</sup> estimated an isotope shift caused by recrystallization and exchange with pore water of  $\sim 0.3 \times 10^{-4}$  in the composition of Miocene calcareous ooze deposited before a 20-Myr increase of  $\sim 5 \times 10^{-4}$  in sea-water  $^{87}\text{Sr}/^{86}\text{Sr}$ . Their modelling indicated that most recrystallization in deep-sea sediment would occur within the first few million

years after deposition. For  $\sim 20$  Myr after deposition of the Bidart Maastrichtian section, the isotopic composition of sea water was fairly constant, creating an insignificant gradient in the isotope ratio in pore water. Moreover, the carbonate in the DSDP section was fine-grained ooze, more chemically reactive than the foraminiferal tests analysed from Bidart, thus further inhibiting the effects of recrystallization relative to the DSDP core<sup>16</sup>. Within experimental error, measured isotopic compositions of Bidart foraminifera agree with published values from DSDP cores covering this same age range<sup>1-4</sup>. Little petrographic or chemical evidence exists for post-diagenetic recrystallization. Oxygen isotope variations of carbonate from the same section at Bidart were interpreted as reflecting the original variations in composition<sup>8</sup>. Changes in measured isotopic composition of strontium are unrelated to fractures and independent of lithologic changes. Finally, foraminifera from these samples show no evidence for recrystallization or diagenetic carbonate overgrowths (W. Orr, personal communication) (Fig. 3).

The change in isotopic composition of the oceans must be a response to processes occurring at least 1.5–2.3 Myr before the K/T boundary, given the assumed age and sedimentation rates for units III to V. Within the sampling resolution of this study, the distinct rise in isotopic composition occurs over a stratigraphic thickness of 30–40 m, or  $\sim 0.75$ –1.0 Myr. The rate of change in the strontium isotope ratio places constraints on the nature and duration of the input. The change in sea water  $^{87}\text{Sr}/^{86}\text{Sr}$  ( $R_{\text{sw}}$ ) with time is  $dR_{\text{sw}}/dt = (R_{\text{in}} - R_{\text{sw}})/\tau$  (refs 17, 18), where  $R_{\text{in}}$  is the  $^{87}\text{Sr}/^{86}\text{Sr}$  composition of Sr supplied to the oceans, and  $\tau$  is its residence time in the oceans. The limiting case of an input of radiogenic Sr into the ocean followed by an instantaneous return to previous conditions would produce an increase in the sea water isotope ratio followed by an exponential decay back to input composition with a time constant of  $1/\tau$ . Assuming the residence time in the oceans was the same in the Cretaceous as today ( $\sim 4$  Myr, ref. 19), the anomalous input of radiogenic Sr to the oceans cannot be

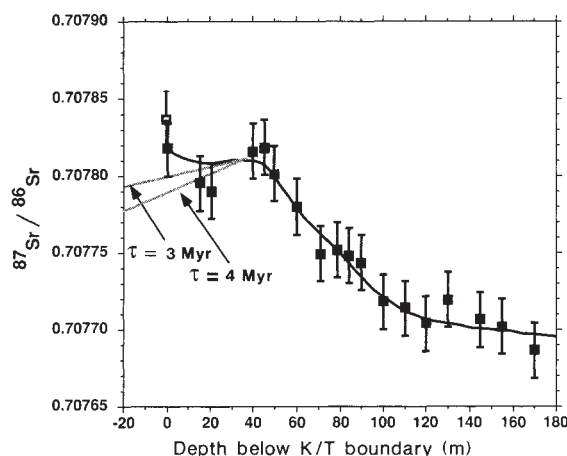


FIG. 2 Average Sr isotopic composition for replicate analyses of each sample of Bidart foraminifera plotted against stratigraphic depth below the K/T boundary. The open symbol is for a sample from the Danian, 5 cm above the boundary. Error bars are  $\pm 26$  p.p.m. Two curves are shown that represent calculated change in Sr isotopic composition of sea water if the conditions of Sr input to the oceans returned to pre-spike values. Curves are shown for assumed Sr residence times ( $\tau$ ) of 3 and 4 Myr, and average depositional rate of  $40 \text{ mm kyr}^{-1}$ . The difference between the model curves and the youngest samples indicates that the anomalous Sr input had to be sustained for 1–2 Myr and was not a geologically instantaneous spike of increased Sr concentration or isotopic composition.

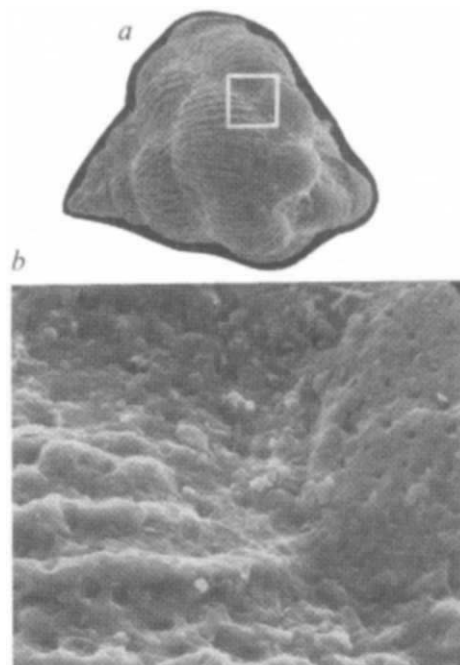


FIG. 3 Scanning electron micrographs of a *Racemiguembelina fruticosa* (Egger) foraminifer test from sample B-74817, 85 m below the K/T boundary at Bidart. The sample is representative of those that record the rapid change in Sr isotopic composition. No carbonate overgrowths were observed on samples from throughout this section (W. Orr, personal communication). The area of micrograph *b* is indicated in *a* by the box which has sides of  $\sim 150 \mu\text{m}$ .



modelled as an instantaneous event or spike, but had to persist from  $\sim 1$  Myr before the Tertiary, until near the K/T boundary; otherwise, the isotopic composition of sea water would have evolved to a value distinctly lower than that observed at the boundary (Fig. 2). A simple mass-balance approach to calculate the amounts of Sr that were delivered to the oceans to generate the observed isotopic change<sup>5,6</sup> indicates that during the period of isotopic increase, the continental Sr influx increased by the equivalent of 4% of the oceanic Sr budget. This calculation assumes a value of 0.712 for  $R_{in}$  (average riverine input of Palmer and Edmond<sup>20</sup> and best estimate of Palmer and Elderfield,<sup>2</sup> for riverine input 65 Myr ago) and no change in mid-ocean ridge hydrothermal activity for 1 Myr. The isotope increase spans  $\sim 40$  m of section, or  $\sim 1$  Myr. Four percent of the ocean Sr budget is  $\sim 10^5$  times the annual riverine input (using present fluxes as an estimate<sup>19</sup>); thus an increased Sr flux of  $\sim 10\%$  over  $10^6$  yr could explain the change in isotopic composition of sea water. It is conceivable that this flux could be provided by increased erosion induced by climate or tectonic changes. A lower flux would be modelled if  $R_{in}$  was more radiogenic than assumed. The rate of change for  $R_{sw}$  in the Late Maastrichtian is  $1.2 \times 10^{-4} \text{ Myr}^{-1}$ . A change of  $0.6\text{--}0.9 \times 10^{-4} \text{ Myr}^{-1}$  was reported for the Early to Middle Miocene<sup>18,21</sup>. Although the rate of change during the Late Maastrichtian is

exceptional, change nearly as rapid is probably not unique to processes that mark the end of the Cretaceous. □

Received 18 January; accepted 9 May 1991.

- Hess, J., Bender, M. L. & Schilling, J.-G. *Science* **231**, 979–984 (1986).
- Palmer, M. R. & Elderfield, H. *Nature* **314**, 526–528 (1985).
- DePaolo, D. J. & Ingram, B. L. *Science* **227**, 938–941 (1985).
- Koepnick, R. B. *et al. Chem. Geol.* **50**, 55–81 (1985).
- Maccougall, J. D. *Science* **239**, 485–487 (1988).
- Javoy, M. & Courtillot, V. *Earth planet. Sci. Lett.* **94**, 409–416 (1989).
- Perch-Nielsen, K., McKenzie, J. & He, Q. *Geol. Soc. Am. Spec. Pap.* **190**, 353–371 (1982).
- Clauser, S. *C.R. heb. Séanc. Sci. Paris* **304**, 579–584 (1987).
- Kent, D. & Gradstein, F. *Geol. Soc. Am. Bull.* **96**, 1419–1427 (1985).
- DePaolo, D. J., Kyte, F. T., Marshall, B. D., O'Neil, J. R. & Smit, J. *Earth planet. Sci. Lett.* **64**, 356–373 (1983).
- MacLeod, K. G. & Ward, P. D. *Geol. Soc. Am. Abstr. Progr.* **22**, 266 (1990).
- Johnson, C. C. & Kauffman, E. G. in *Extinction Events in Earth History* (eds Kauffman, E. G. & Walliser, O. H.) 305–307 (Springer, Berlin, 1990).
- Officer, C. B. & Drake, C. L. *Science* **227**, 1161–1167 (1985).
- Richter, F. M. & DePaolo, D. J. *Earth planet. Sci. Lett.* **83**, 27–38 (1987).
- Richter, F. M. & DePaolo, D. J. *Earth planet. Sci. Lett.* **90**, 382–394 (1988).
- DePaolo, D. J. & Finger, K. L. *Geol. Soc. Am. Bull.* **103**, 112–124 (1991).
- Brass, G. W. *Geochim. cosmochim. Acta* **40**, 721–730 (1976).
- DePaolo, D. J. *Geology* **14**, 103–106 (1986).
- Broecker, W. S. & Peng, T.-H. *Tracers in the Sea*, 1–690 (Lamont-Doherty Geological Observatory, Palisades, 1982).
- Palmer, M. R. & Edmond, J. M. *Earth planet. Sci. Lett.* **92**, 11–26 (1989).
- Hodell, D. A., Mueller, P. A. & Garrido, J. R. *Geology* **19**, 24–27 (1991).

ACKNOWLEDGEMENTS. We thank W. Orr for providing Fig. 3 and N. H. M. Swinburne for discussion and a detailed review of the manuscript.

## Direct measurement of active dispersal of food-falls by deep-sea demersal fishes

I. G. Priede, P. M. Bagley, J. D. Armstrong, K. L. Smith Jr\* & N. R. Merrett†

Department of Zoology, University of Aberdeen, Tillydrone Avenue, Aberdeen AB9 2TN, UK

\* Marine Biology Research Division, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, California 92093, USA

† Department of Zoology, The Natural History Museum, Cromwell Road, London SW7 5BD, UK

BAITED cameras on the deep ocean floor first revealed the presence of communities of scavengers, including deep demersal fishes capable of consuming food falls and thus dispersing surface-derived organic carbon<sup>1–3</sup>. By embedding acoustic transmitters in baits and deploying them together with an automatic tracking system and cameras on the sea floor<sup>4,5</sup>, we have now tracked the speeds and directions of departing deep demersal scavenging fishes. At a series of stations between 4,000 and 6,000 m deep in the Northern Hemisphere, in contrasting trophic regimes, we have found that two closely related species of fish, *Coryphaenoides* (*Nematonurus*) *armatus* and *C.(N.) yaquinae* have a significant role in bait dispersal. Even in remote oligotrophic locations, transmitters were consumed rapidly and were removed from the area of detection at a mean velocity of  $0.11 \text{ m s}^{-1}$ . We find that these fish are active foragers, constantly moving independently of bottom currents. This result is contrary to previous speculation of passive or drifting strategies<sup>6</sup> which might have been expected to conserve energy in a food-limiting environment.

Five locations were studied (Table 1) in the North Atlantic and Pacific oceans. These varied from relatively eutrophic areas (2–4) of high seasonal productivity<sup>4</sup> to oligotrophic areas in the central North Pacific (area 1) and North Atlantic (area 5), the latter having a disturbed sea floor<sup>7</sup> with very low benthic biomass. Baits with acoustic transmitters were deployed beneath a free-fall vehicle which was programmed to record data autonomously before rising to the sea surface for recovery by a surface ship<sup>5</sup>. The vehicle carried instruments for recording

current direction and in some cases velocity close to the sea floor. Bait was one whole mackerel per deployment with up to three transmitters per deployment.

Fish arrived at the baits swimming up-current following the odour plume emanating from the bait<sup>6,8,9</sup>. Assuming an inverse square relationship<sup>4</sup>, the theoretical fish population density was calculated from the time delay of fish arrival (Table 1). For

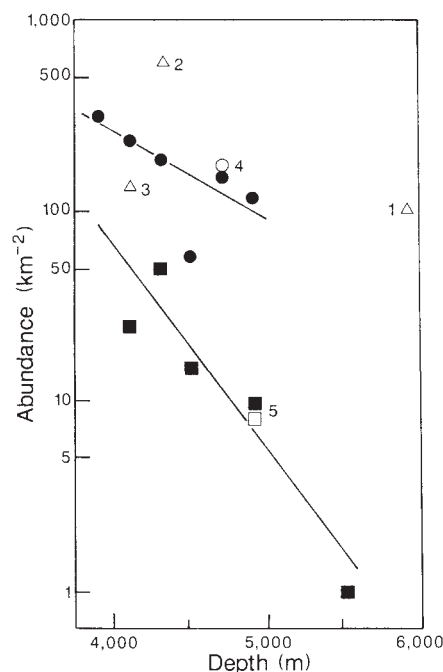


FIG. 1 Estimates of abundance of *C.(N.) armatus* in the North Atlantic. ●, Trawl data for latitudes  $41^{\circ}$ – $50^{\circ}$ N, areas of high seasonal production; ■, trawl data for latitudes  $20^{\circ}$ – $38^{\circ}$ N, areas of low production. Numbered symbols refer to theoretical values based on fish arrival times at baits listed in Table 1. △, Stations 1, 2 and 3 in the North Pacific. ○ and □, Stations 4 and 5 in the North Atlantic; note the close correlation with corresponding trawl data.

TABLE 1 Grenadier fish, first arrival times, estimated population density and staying times at stations in the northern Atlantic and Pacific Oceans

| Station | Ocean (depth)      | Name                    | Position lat./long.   | Production | First fish arrival (min) | Fish density (km <sup>-2</sup> ) | Staying time (min) |
|---------|--------------------|-------------------------|-----------------------|------------|--------------------------|----------------------------------|--------------------|
| 1       | Pacific (5,900 m)  | CNP                     | 31° N, 159° W         | Low        | 38                       | 105                              | 261                |
| 2       | Pacific (4,400 m)  | Station F               | 32° 50' N, 124° W     | High       | 16                       | 601                              | 60                 |
| 3       | Pacific (4,100 m)  | Station M               | 32° 50' N, 122° 50' W | High       | 34                       | 132                              | 113                |
| 4       | Atlantic (4,800 m) | Porcupine Abyssal Plain | 48° 50' N, 16° 50' W  | High       | 30                       | 167                              | 141                |
| 5       | Atlantic (4,900 m) | Madeira Abyssal Plain   | 31° N, 21° W          | Low        | 138                      | 8                                | 364                |
| Means   |                    |                         |                       |            | 51                       | 203                              | 188                |

comparison, samples were taken near the two stations in the North Atlantic (Fig. 1) using semiquantitative trawling techniques<sup>10</sup>. Abundance estimates correlated well with the theoretical values for stations 4 and 5. No trawl samples were taken from the North Pacific but theoretical abundance was similar to the high latitude data from the North Atlantic. It is evident that, except at station 5, several hundred fish can be expected to be present within the 1 km range of detection of the tracking system. Mean biomass of *C.(N.) armatus* in the Atlantic trawls was 333 kg km<sup>-2</sup> at station 4 and 16.8 kg km<sup>-2</sup> at station 5.

Two species of grenadier were observed feeding at baits; *C.(N.) armatus* in the Atlantic and Pacific oceans (stations 2–5) and *C.(N.) yaquinae* in the central North Pacific (station 1). In the Atlantic a few individuals of other species of fish were observed, for example, *Synaphobranchus bathybius*. In abyssal trawl samples in the North Atlantic, *C.(N.) armatus* comprised 14–65% (mean 38%) of the catch at station 4 and 0–15% (mean 2.6%) at station 5, and yet represented more than 90% of the fish observed at baits. *C.(N.) armatus* is the most ubiquitous of all deep demersal fishes<sup>11</sup>, whereas *C. yaquinae* is the replacement counterpart in the deep Pacific (>4,300 m)<sup>12</sup> but does not occur in the Atlantic.

Transmitters were ingested at a mean time of 103 min after touchdown on the sea floor (s.d. = 98.4 min). Fish that ingested transmitters moved away from the bait source (Fig. 2), the fitted line indicating a mean departure time of 194 min after touchdown on the sea floor and mean radial velocity of 0.116 m s<sup>-1</sup>. No transmitters remained within range of the receiving system after 24 h, most departing much earlier, so there is no evidence

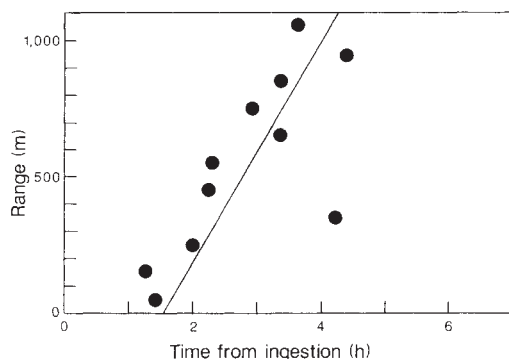


FIG. 2 Ranges of fish in relation to mean time (0) after ingestion of acoustic transmitters based on 1,859 measurements from 55 fish. Mean ingestion time is 103 min (s.d. = 94.85) after the bait reached the sea floor, 63 observations: not all fish observed ingesting transmitters were tracked successfully. Data points shown are mean times at 100 m range intervals. The intercept on the time axis, 91.2 min., is the mean time of departure from near the bait. The slope of the line indicates a mean velocity thereafter of 0.116 m s<sup>-1</sup>.

for territorial or home-range behaviour in these fishes. The mean velocity is higher than the mean tidal current velocities on the sea floor which vary between 0 and 0.1 m s<sup>-1</sup> (ref. 4) during the tidal cycle. Fish could achieve these speeds by swimming with favourable currents as do certain shallow-water fishes<sup>13</sup>, but analysis of vanishing bearings relative to the current (Fig. 3) shows a wide dispersal of angles with significant bias towards cross-current movement. The hypotheses that these fish are passive drifters or use selective tidal stream transport are thus rejected.

An individual grenadier has the potential to move about 3,000 km yr<sup>-1</sup> at the velocities recorded, independent of currents. Material may thus be dispersed throughout the ocean basins. *C.(N.) armatus* has a pandemic distribution occurring in all zones deeper than 2,000 m examined in a recent review of the North Atlantic basin<sup>11</sup>. The almost exclusive dominance of *C.(N.) armatus* spp. is unexpected. Normally in baited camera studies the bait is protected inside a mesh or protective container to prolong the attractant effect of the odour plume. In such cases a succession of fish species may be observed and invertebrates, notably amphipods, burrow into the bait<sup>2,8</sup>. In the present work the bait was unprotected and susceptible to rapid consumption by dominant large scavengers. These first fish attracted to the baits represent only a fraction of deep ichthyofauna and other species may use less active foraging strategies. Even *C.(N.) armatus* do not feed exclusively on surface-derived material, stomach contents typically contain a wide range of organisms<sup>14</sup>. The consumption of food falls is part of an active opportunistic foraging strategy. The active foraging mode of *C.(N.) armatus* seems to be at variance with the observation that this species has a low metabolic rate compared with shallow-water fishes<sup>15</sup> so might be expected to adopt less active, energy-saving behaviours. These fish seem to be working in accordance with the predictions of the optimal foraging marginal value

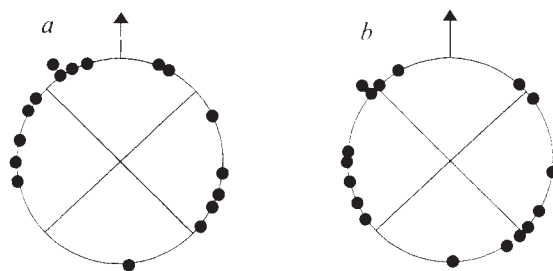


FIG. 3 a, Vanishing angles at the 1 km range of 17 *C.(N.) armatus* (12 fish from station 5 and five from station 3). Angles are relative to the bottom current direction (arrow). Rao's and Rayleigh's tests<sup>17</sup> indicate no preferred direction in this data. b, Data from (a) but with the mean current vector subtracted. There is a significant bias ( $\chi^2$  test) to the cross-current sectors ( $P > 0.05$ ) compared with coaxial sectors.



theorem<sup>16,4</sup>. Fish in food-sparse environments 1 and 5 (Table 1) stay at the bait source longer than in the more food-rich environments.

This study shows that large organic falls reaching the sea floor are rapidly dispersed at abyssal depths in the Northern Hemisphere. There are significant spatial variations in departure rates depending on abundance of scavengers and prevailing food availability. The range of these demersal fish on a basin-wide scale must be considered when modelling the distribution and fate of natural and anthropogenic inputs to the deep ocean. □

Received 19 April; accepted 17 May 1991.

1. Isaacs, J. D. & Schwartzlose, R. A. *Scient. Am.* **233**, 85–91 (1975).
2. Thurston, M. *Mar. Biol.* **51**, 55–68 (1979).
3. Stockton, W. L. & Delaca, T. E. *Deep Sea Res.* **29**, 157–169 (1982).
4. Priede, I. G., Smith, K. L. & Armstrong, J. D. *Deep Sea Res.* **37**, 81–101 (1990).
5. Bagley, P., Priede, I. G. & Armstrong, J. D. *Institute of Electrical Engineers Colloquium* 182 (1990).
6. Wilson, R. R. & Smith, K. L. *Mar. Biol.* **84**, 83–91 (1984).
7. Weaver, P. E. & Kuipers, A. *Nature* **306**, 360–363 (1983).
8. Desbruyeres, D., Geistdoerfer, P., Ingram, C. L., Khripounoff, A. & Lagardere, J. P. in *Peuplements Profonds du Golfe de Gascogne* (eds Laubier, L. & Monniot, C.) 233–251 (IFREMER, Brest, 1985).
9. Sainte-Marie, E. & Hargrave, B. T. *Mar. Biol.* **94**, 431–443 (1987).
10. Merrett, N. R., Haedrich, R. L., Gordon, J. D. M. & Stehman, M. J. *Mar. Biol. Ass. U.K.* **71**, 359–373 (1991).
11. Haedrich, R. L. & Merrett, N. R. *J. nat. Hist.* **22**, 1325–1362 (1988).
12. Wilson, R. R. & Waples, R. S. *Deep Sea Res.* **30**, 1127–1145.
13. Greer-Walker, M., Harden-Jones, F. R. & Arnold, G. P. *J. Cons. perm. int. Explor. Mer* **38**, 58–93 (1978).
14. Mauchline, J. & Gordon, J. D. M. *Mar. Biol.* **81**, 107–121 (1984).
15. Smith, K. L. *Nature* **274**, 362–364 (1978).
16. Stephens, D. W. & Krebs, J. R. *Foraging Theory* (Princeton University Press, New Jersey, 1986).
17. Batschelet, E. *Circular Statistics in Biology* (Academic, New York, 1981).

ACKNOWLEDGEMENTS. We thank colleagues onshore and on board the research vessels in the Pacific and Atlantic oceans and M. Thurston for critical reading of this manuscript. The work was supported by the Wolfson Foundation, NSF and NERC.

## Is the guinea-pig a rodent?

Dan Graur\*†, Winston A. Hide†‡ & Wen-Hsiung Li†§

\* Department of Zoology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv 69978, Israel

† Center for Demographic and Population Genetics, University of Texas, PO Box 20334, Houston, Texas 77225, USA

‡ Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030, USA

**THE guinea-pig (*Cavia porcellus*), traditionally classified as a New World hystricomorph rodent, often shows anomalous morphological and molecular features in comparison with other eutherian mammals<sup>1–14</sup>. For example, its insulin differs from that of other mammals in anabolic and growth-promoting activities and in its capability to form hexamers<sup>5,6</sup>. Indeed, the literature about the molecular evolution of guinea-pigs abounds in references to ‘convergent evolution’, ‘extremely rapid rates of substitution’, and ‘unique evolutionary mechanisms’. These claims are based on the assumption that the guinea-pig is a rodent. Our phylogenetic analyses of amino-acid sequence data, however, imply that the guinea-pig diverged before the separation of the primates and the artiodactyls from the myomorph rodents (rats and mice). If true, then the myomorphs and the caviomorphs do not constitute a natural clade, and the Caviomorpha (or the Hystricomorpha) should be elevated in taxonomical rank and regarded as a separate mammalian order distinct from the Rodentia. If, as suggested by recent data<sup>15,16</sup>, the myomorphs branched off before the divergence among the carnivores, lagomorphs, artiodactyls and primates, then the new order would represent an early divergence in eutherian radiation.**

The Rodentia, which is the most speciose mammalian order, is traditionally divided into three extant suborders: the Sciuromorpha (squirrel-like rodents), the Myomorpha (rat-like

rodents) and the Hystricomorpha (porcupine-like rodents)<sup>1</sup>. Whereas the first two suborders are generally considered to be monophyletic, the New World families of the Hystricomorpha (the Caviomorpha or guinea-pig-like rodents) are sometimes thought to have evolved independently of the Old World hystricomorph rodents, and are, thus, regarded as a separate group within the Rodentia<sup>2</sup>. Claims about the peculiarity of guinea-pig genes are usually based on the assumption that the guinea-pig is indeed a rodent. A different taxonomic assignment may resolve or ameliorate many of the paradoxes associated with the evolution of guinea-pig genes.

To infer the taxonomic position of the guinea-pig, we analysed the phylogeny of protein sequence data from the caviomorphs, myomorphs, the primates, the artiodactyls and some outgroup species such as marsupials, birds, or toads. We used the maximum parsimony method<sup>17</sup> and the PROTPARS program of Felsenstein's PHYLIP package (version 3.3) to calculate the number of amino-acid replacements required for each of the alternative trees, and the number of informative sites supporting each of the trees.

We first consider, besides the outgroup, only three eutherian groups: the guinea-pig (or caviomorphs), the myomorphs and the primates. This simplifies the analysis because the branching order of eutherians is not well established. Moreover, it increases the amount of data for analysis because there are more sequence data for the primates than for other orders. If there are more than one sequence available in a group, we choose sequences with known branching order. For example, for the primates we choose the human, an Old World monkey (the rhesus monkey,

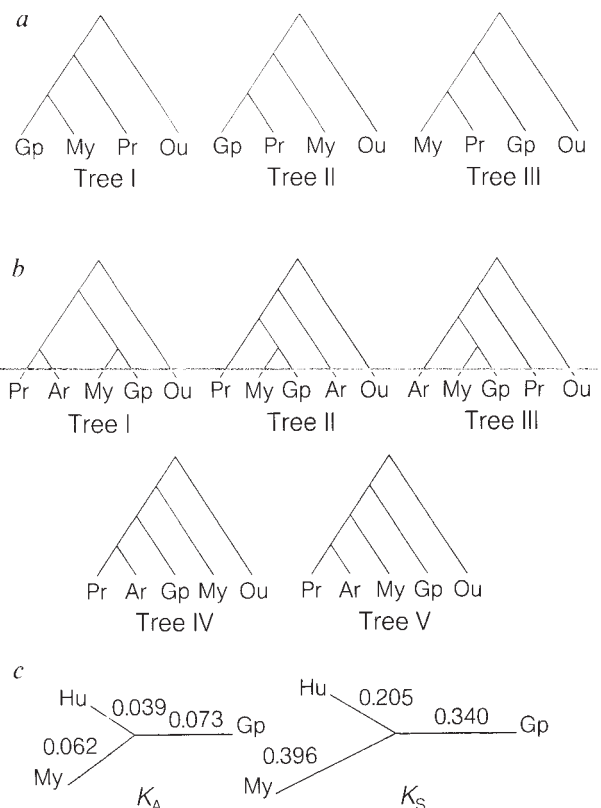


FIG. 1 a, Three possible phylogenetic trees for guinea-pigs (Gp), primates (Pr), myomorphs (My), and an outgroup (Ou). Tree I represents the traditional view that the guinea-pig and the myomorphs form one clade. b, Five of 15 possible alternative phylogenetic trees for primates, artiodactyls (Ar), myomorphs, guinea-pigs, and an outgroup. c, Branch lengths from a common node to humans (Hu), myomorphs (My) and guinea-pigs (Gp) computed from the number of nucleotide substitutions per nonsynonymous site ( $K_A$ ) and per synonymous site ( $K_S$ ) given in Table 3.

§ To whom correspondence should be addressed.

TABLE 1 Number of informative sites supporting each of the three possible alternative phylogenetic trees (Fig. 1a), and minimum number of amino-acid replacements required for each tree (in parentheses)

| Protein                           | Out-group | Primates (Pr), guinea-pig (Gp) and myomorphs (My) |           |                | Artiodactyls (Ar), guinea-pig (Gp) and myomorphs (My) |                  |       | L           | I                |                 |                  |  |
|-----------------------------------|-----------|---------------------------------------------------|-----------|----------------|-------------------------------------------------------|------------------|-------|-------------|------------------|-----------------|------------------|--|
|                                   |           | L*                                                | I†        | Tree I (Gp-My) | Tree II (Gp-Pr)                                       | Tree III (Pr-My) | L     | I           | Tree I (Gp-My)   | Tree II (Gp-Ar) | Tree III (Ar-My) |  |
| $\beta$ -globin                   | MA        | 145                                               | 9         | 5 (189)        | 0 (194)                                               | 4 (189)          | 145   | 11          | 7 (210)          | 0 (217)         | 4 (213)          |  |
| $\alpha$ -crystallin A chain      | MA        | 162                                               | 1         | 0 (33)         | 1 (32)                                                | 0 (33)           | 162   | 1           | 1 (29)           | 0 (30)          | 0 (30)           |  |
| $\alpha$ -globin                  | MA        | 145                                               | 9         | 4 (170)        | 0 (174)                                               | 5 (168)          | 145   | 9           | 2 (190)          | 1 (191)         | 6 (185)          |  |
| $\alpha$ -lactalbumin             | MA        | 120                                               | 17        | 4 (148)        | 8 (143)                                               | 5 (147)          | 120   | 12          | 1 (188)          | 6 (183)         | 5 (184)          |  |
| glucagon                          | MA        | 29                                                | 0         | 0 (5)          | 0 (5)                                                 | 0 (5)            | 29    | 0           | 0 (5)            | 0 (5)           | 0 (5)            |  |
| pancreatic ribonuclease           | MA        | 122                                               | 7         | 1 (193)        | 5 (189)                                               | 1 (193)          | 122   | 5           | 2 (223)          | 3 (222)         | 0 (225)          |  |
| 'big' gastrin                     | MA        | 31                                                | 1 + gap   | 0 (22)         | 0 (22)                                                | 1 + gap (21)     | 31    | 1 + gap     | 0 (28)           | 0 (28)          | 1 + gap (27)     |  |
| adrenocorticotrophin              | OS        | 39                                                | 1         | 0 (11)         | 0 (11)                                                | 1 (10)           | 39    | 1           | 0 (13)           | 0 (13)          | 1 (12)           |  |
| lipocortin                        | PG        | 345                                               | 19        | 4 (179)        | 4 (179)                                               | 11 (172)         | 25    | 2           | 1 (31)           | 1 (31)          | 0 (32)           |  |
| pancreatic polypeptide            | CK        | 36                                                | 0         | 0 (15)         | 0 (15)                                                | 0 (15)           | 36    | 1           | 0 (38)           | 1 (37)          | 0 (38)           |  |
| proinsulin                        | CK        | 78                                                | 4         | 1 (74)         | 1 (74)                                                | 2 (73)           | 78    | 4           | 2 (87)           | 0 (89)          | 2 (87)           |  |
| lipoprotein lipase                | CK        | 447                                               | 20        | 2 (206)        | 6 (202)                                               | 12 (196)         | 447   | 21          | 2 (218)          | 9 (212)         | 10 (211)         |  |
| $\beta$ -nerve growth factor      | CK        | 171                                               | 11        | 5 (84)         | 1 (88)                                                | 5 (84)           | 123   | 7           | 5 (38)           | 0 (43)          | 2 (41)           |  |
| vasoactive intestinal peptide     | CK        | 28                                                | 1         | 0 (8)          | 0 (8)                                                 | 1 (7)            | 28    | 1           | 0 (8)            | 0 (8)           | 1 (7)            |  |
| vasopressin-neurophysin precursor | TD        | 100                                               | 2         | 0 (60)         | 2 (58)                                                | 0 (60)           | 102   | 1 + gap     | 1 + gap (58)     | 0 (59)          | 0 (59)           |  |
| Total                             |           | 1,998                                             | 102 + gap | 26 (1,397)     | 28 (1,394)                                            | 48 (1,373) + gap | 1,632 | 77 + 2 gaps | 24 (1,364) + gap | 21 (1,368)      | 32 (1,356) + gap |  |

Sequences in this and other tables from the GenBank, EMBL, NBRF-PIR, and Swiss Prot DNA or protein data libraries. The outgroup species used are CK, chicken; OS, ostrich; PG, pigeon; TD, toad (*Bufo japonicus*); and MA, marsupials. Opossum (*Didelphis virginiana*) and red kangaroo (*Macropus rufus*) used for  $\beta$ -globin and  $\alpha$ -crystallin A chain; opossum and Eastern grey kangaroo (*M. giganteus*) for  $\alpha$ -globin; red-necked wallaby (*M. rufogriseus*) for  $\alpha$ -lactalbumin; opossum for glucagon and 'big' gastrin; and red kangaroo for pancreatic ribonuclease. For the caviomorph group, guinea-pig is used in all cases, except that chinchilla, cuis, and capybara are used for pancreatic ribonuclease and guinea-pig and chinchilla are used for 'big' gastrin. For the other three eutherian groups the species used are: human, rhesus monkey, spider monkey, mouse, rat, golden hamster, cow, goat, and Bactrian camel (*Camelus bactrianus*) for the  $\beta$ -globin; human, rhesus monkey, galago (bush baby), mouse, rat, golden hamster, cow, camel (*Camelus dromedarius*) and pig for  $\alpha$ -crystallin A chain; human, baboon, spider monkey, mouse, rat, golden hamster, cow, camel and pig for  $\alpha$ -globin; human, rat, cow, goat and camel for  $\alpha$ -lactalbumin; human, rat and pig for glucagon; human, mouse, rat, golden hamster, cow, camel, and giraffe for pancreatic ribonuclease; human, rat, cow and pig for 'big' gastrin; human, rat, cow and pig for pancreatic polypeptide; human, mouse, rat, cow and pig for adrenocorticotrophin; human, rat and pig for lipocortin; human, mouse, rat, cow and pig for proinsulin; human, mouse and cow for lipoprotein lipase; human, mouse and cow for  $\beta$ -nerve growth factor; human, rat and pig for vasoactive intestinal peptide; human, rat, cow and pig for vasopressin-neurophysin precursor. Informative sites are all those amino-acid positions at which the number of substitutions required differs among the possible alternative phylogenetic trees. An informative site is said to support a tree if that tree requires the least number of substitutions at that site in comparison with the alternative possible trees. Note that the number of informative sites supporting trees I and II are 26 and 28, but tree I requires 3 additional substitutions. This is because replacement of an amino acid by another at a certain site may sometimes require more than one nonsynonymous nucleotide substitution at the DNA level.

\* L, number of aligned amino-acid sites.

† I, number of informative sites.

for instance), and a New World monkey (spider monkey). The species used are given in Table 1. With these three groups and an outgroup (which may comprise more than one species) there are three possible phylogenetic trees (Fig. 1a). Tree I represents the presently accepted taxonomic scheme, in which the guinea-pig and the myomorphs are clustered to form a clade. In tree II, the guinea-pig and the primate lineages form one clade, with the two groups more closely related to each other than either is to the myomorphs. In tree III, the guinea-pig is an outgroup to both primates and myomorphs.

Table 1 presents the results of this analysis for 15 protein sequences with a total of 1,998 aligned amino-acid sites. There are 102 informative sites, of which 48 sites support tree III, whereas only 26 and 28 sites support trees I and II, respectively. The probability that tree III is wrong is roughly  $P < 0.05$  (refs 18, 19); the test is rough because it assumes rate-constancy and equal weights for all informative sites. (Although 48% of the sites supporting tree III are due to lipocortin and lipoprotein lipase, this is not much higher than the contribution (40%) of these two proteins to the 1,998 sites analysed.) The molecular evidence against the traditional view (tree I) is very strong, for it is supported by only 26 of the 102 informative sites. The amino-acid sequence of gastrin<sup>8</sup> provides additional support for tree III. The 'big' gastrins of dogs, cats, cows, sheep, goats, humans, rats and rabbits are of 34 amino acids. In comparison, the 'big' gastrins of two New World hystricomorphs, guinea-pig

and chinchilla (*Chinchilla brevicaudata*), are of only 33 amino acids, lacking a glutamic acid near their terminus. The same feature was noted in the gastrin of opossum (*Didelphis virginiana*), a marsupial.

TABLE 2 Number of amino-acid differences between an outgroup (OU) and humans (HU), myomorphs (MY) and guinea-pigs (GP)

| Protein                           | OU-HU | OU-MY | OU-GP |
|-----------------------------------|-------|-------|-------|
| Outgroup: marsupial               |       |       |       |
| $\alpha$ -globin                  | 27    | 33    | 36    |
| pancreatic ribonuclease           | 42    | 42    | 46    |
| $\alpha$ -lactalbumin             | 55    | 66    | 66    |
| $\alpha$ -crystallin              | 16    | 14    | 15    |
| $\beta$ -globin                   | 38    | 45    | 44    |
| glucagon                          | 2     | 2     | 6     |
| 'big' gastrin                     | 6     | 10    | 10    |
| pancreatic polypeptide            | 5     | 10    | 5     |
| Total                             | 191   | 222   | 226   |
| Outgroup: chicken                 |       |       |       |
| $\beta$ nerve growth factor       | 71    | 80    | 74    |
| lipoprotein lipase                | 104   | 104   | 122   |
| vasoactive intestinal polypeptide | 4     | 4     | 6     |
| insulin                           | 41    | 37    | 51    |
| Total                             | 220   | 225   | 253   |



TABLE 3 Number of substitutions per nonsynonymous site ( $K_A$ ) and synonymous site ( $K_S$ ) between guinea-pigs and humans (GP-HU), guinea-pigs and myomorphs (GP-MY), and humans and myomorphs (HU-MY)

| Gene                         | N     | $K_A$ |       |       | $K_S$ |       |       |
|------------------------------|-------|-------|-------|-------|-------|-------|-------|
|                              |       | GP-HU | GP-MY | HU-MY | GP-HU | GP-MY | HU-MY |
| preproinsulin                | 330   | 0.208 | 0.211 | 0.102 | 0.700 | 0.854 | 0.673 |
| apolipoprotein E             | 906   | 0.149 | 0.176 | 0.175 | 0.387 | 0.771 | 0.618 |
| preproglucagon               | 540   | 0.044 | 0.061 | 0.043 | 0.464 | 0.660 | 0.560 |
| $\alpha$ -lactalbumin        | 411   | 0.170 | 0.241 | 0.174 | 0.550 | 1.112 | 0.848 |
| lipoprotein lipase           | 1,335 | 0.065 | 0.071 | 0.029 | 0.653 | 0.671 | 0.607 |
| pancreatic polypeptide       | 213   | 0.149 | 0.193 | 0.155 | 0.958 | 0.789 | 0.486 |
| N-ras proto-oncogene         | 564   | 0.002 | 0.009 | 0.007 | 0.284 | 0.516 | 0.462 |
| factor IX                    | 804   | 0.116 | 0.125 | 0.097 | 0.366 | 0.676 | 0.525 |
| insulin-like growth factor I | 604   | 0.210 | 0.292 | 0.224 | 0.861 | 0.846 | 0.666 |
| Weighted average             |       | 0.112 | 0.136 | 0.101 | 0.545 | 0.736 | 0.601 |

Next, we replace the primate group by the artiodactyl group. The data set now becomes smaller (1,632 aligned sites) and the number of informative sites is reduced to 77. The number of sites supporting tree III is 32 whereas those supporting trees I and II are only 24 and 21, respectively. Therefore, the result again supports the view that the guinea-pig has branched off earlier than did the myomorphs.

Finally, we consider the guinea-pig, the myomorphs, the primates and the artiodactyls together. The first three trees in Fig. 1b represent the traditional view that the guinea-pig and the myomorphs belong to one clade. We compare these trees with tree V, which is suggested by the above analyses and the study of Li *et al.*<sup>16</sup>, and also with tree IV, which is a modification of tree V with the order of My and Gp reversed. The minimum numbers of amino-acid replacements required for each of the five trees are 1,460, 1,457, 1,458, 1,457, and 1,442. Thus, tree V requires at least 15 steps fewer than trees I, II and III, which are only as parsimonious as tree IV. In this analysis, the sites used are the same as those used in the second analysis of Table 1, but the differences in steps between the most parsimonious tree and the other trees have become larger as a result of including the primate group. The result strengthens our hypothesis that the guinea-pig branched off earlier than the myomorphs and also the suggestion of Li *et al.*<sup>16</sup> that the myomorphs are an outgroup to the primates and artiodactyls.

Thus our analyses suggest that the guinea-pig represents a separate evolutionary lineage from the myomorph rodents, and should not be classified in the same order. It is not yet possible to decide which species besides the caviomorphs belong to the new mammalian order represented by the guinea-pig. Although controversial, the caviomorphs are commonly regarded as a subgroup of the hystricomorphs, and this is supported by limited molecular data<sup>20</sup>. We therefore tentatively suggest that the hystricomorphs represent a new order.

A new taxonomic assignment for the guinea-pig resolves or ameliorates many of the paradoxes associated with the evolution of its genes. For example, consider lipocortin. Under the assumption that the guinea-pig is a rodent or at least a sister group of the rodents, one must assume that at least 15 amino-acid replacements have occurred in parallel in pigeons and in guinea-pigs. The number of parallel substitutions is reduced to 8 if the new taxonomic position of the guinea-pig is accepted.

In many studies the rate of substitution in guinea-pig genes relative to that in myomorph species has been computed by using the relative rate test<sup>21</sup> with human or bovine genes as an outgroup; that is, tree I in Fig. 1a is assumed to represent the true phylogeny. For many genes the conclusion was that the guinea-pig lineage evolves much faster than the myomorph lineage. For example, the  $\alpha$ - and  $\beta$ -globin chains evolved roughly two to four times faster in the guinea-pig than in mice or rats<sup>22</sup>. By contrast, under our proposed phylogenetic position for the guinea-pig, the two sequences seem to have evolved at about the same rate in both the caviomorph and the myomorph

lineages. Table 2 lists the number of amino-acid differences between an outgroup, on the one hand, and humans, myomorphs or guinea-pigs, on the other. For the group of proteins for which the outgroup is a marsupial, there seems to be no difference in the number of replacements between myomorphs and guinea-pigs. For the group of proteins for which chicken serves as the outgroup, the guinea-pig seems to have accumulated an excess of amino-acid replacements in comparison to myomorphs. The excess, however, can be entirely attributed to insulin and lipoprotein lipase, both of which seem to have evolved much faster in the guinea-pig than in other mammals (refs. 5, 6, 23; W.A.H. and W.-H.L., unpublished results). We therefore conclude that claims of large differences in the rate of evolution between guinea-pigs and myomorphs may have been exaggerated in many cases as a result of an erroneous phylogenetic assumption. But there is evidence that both the caviomorph and myomorph lineages have evolved faster than the human lineage (Table 2).

Several DNA sequences are available for computing the genetic distance between guinea-pigs, on the one hand, and myomorphs or humans, on the other (Table 3). The number of substitutions per nonsynonymous site ( $K_A$ ) and per synonymous site ( $K_S$ ) were estimated by the method of Li *et al.*<sup>24</sup>. In almost all cases, the values of both  $K_A$  and  $K_S$  between guinea-pigs and humans are smaller than the corresponding values between guinea-pigs and myomorphs. The only exceptions are the  $K_S$  values for pancreatic polypeptide and insulin-like growth factor I. Therefore, even if tree I in Fig. 1a represents the true branching order, the genetic distances between guinea-pigs and myomorphs are large enough to warrant a separate ordinal status for the Caviomorpha. Again, the results indicate that myomorph genes evolve 1.5–2.0 times faster than human genes (Fig. 1c).

The proposed new evolutionary position of the guinea-pig needs to be re-examined in the future using more sequence data, particularly by replacing those bird sequences by marsupial sequences as references. If the hypothesis turns out to be false, and the guinea-pig is indeed a rodent, then the genetic distances in Table 3 imply a much faster rate of nucleotide substitution in guinea-pig and myomorph genes than in human genes and the data in Table 1 provide a dramatic example that unequal rates of evolution can consistently mislead parsimony inferences<sup>25</sup>, even when the characters are from amino-acid sequences. □

Received 22 January; accepted 3 May 1991

- Nowak, R. M. & Paradiso, J. L. *Walker's Mammals of the World* (Johns Hopkins University Press, Baltimore, 1983).
- Romer, A. S. *Notes and Comments on Vertebrate Paleontology* (University of Chicago Press, 1968).
- Sahni, A. in *Evolutionary Relationships among Rodents: A Multidisciplinary Analysis* (eds Luckett, W. P. & Hartenberger, J.-L.) 133–150 (Plenum, New York, 1985).
- Wood, A. E. in *Evolutionary Relationships among Rodents: A Multidisciplinary Analysis* (eds Luckett, W. P. & Hartenberger, J.-L.) 475–509 (Plenum, New York, 1985).
- Beintema, J. J. & Campagne, R. N. *Molec. Biol. Evol.* **4**, 10–18 (1987).
- Watt, V. M. *J. Biol. Chem.* **260**, 10926–10929 (1985).
- Manao, G. *et al. J. Prot. Chem.* **7**, 417–426 (1988).

8. Shinomura, Y., Eng, J., Rattan, S. C. & Yalow, R. S. *Comp. Biochem. Physiol.* **96B**, 239–242 (1990).
9. Beintema, J. J. & Neuteboom, B. *J. molec. Evol.* **19**, 145–152 (1983).
10. Sarkar, G., Koeberl, D. D. & Sommer, S. S. *Genomics* **6**, 133–143 (1990).
11. Fan, Z.-W., Eng, J., Shaw, G. & Yalow, R. S. *Peptides* **9**, 429–431 (1988).
12. Smith, A. I. *et al.* *J. Endocr.* **115**, R5–R8 (1987).
13. Wolfe, P. B. & Cebra, J. J. *Molec. Immun.* **17**, 1493–1505 (1980).
14. Eng, J., Du, B.-H., Raufman, J.-P. & Yalow, R. S. *Peptides* **7**, Suppl. 1, 17–20 (1986).
15. Easteal, S. *Genetics* **124**, 165–173 (1990).
16. Li, W.-H., Gouy, M., Sharp, P. M., O'Huigin, C. & Yang, Y.-W. *Proc. natn. Acad. Sci. U.S.A.* **87**, 6703–6707 (1990).
17. Fitch, W. M. *Am. Nat.* **116**, 111–120 (1980).
18. Felsenstein, J. *Syst. Zool.* **34**, 152–161 (1985).
19. Li, W.-H. & Gouy, M. *Meth. Enzym.* **183**, 645–659 (1990).
20. Fitch, W. M. & Beintema, J. J. *Molec. Biol. Evol.* **7**, 438–443 (1990).
21. Sarich, V. M. & Wilson, A. C. *Science* **158**, 1200–1203 (1973).
22. Shoshani, J., Goodman, M., Czelusniak, J. & Braunitzer, G. in *Evolutionary Relationships among Rodents: A Multidisciplinary Analysis* (eds Luckett, W. P. & Hartenberger, J.-L.) 191–210 (Plenum, New York, 1985).
23. Yu, J.-H., Eng, J., Rattan, S. & Yalow, R. S. *Peptides* **10**, 1195–1197 (1989).
24. Li, W.-H., Wu, C.-I. & Luo, C.-C. *Molec. Biol. Evol.* **2**, 150–174 (1985).
25. Felsenstein, J. *Syst. Zool.* **27**, 401–415 (1978).

ACKNOWLEDGEMENTS. We thank M. Gouy, R. Honeycutt, H. Mendelssohn, T. Mendelson, D. Eilam, Z. Sever, and L. Jacobs for help and suggestions and J. Felsenstein for sending us the PHYLIP package. This study was supported by NIH (W.-H.L.) and the US-Israel Binational Science Foundation (D.G.).

## Adaptive protein evolution at the *Adh* locus in *Drosophila*

John H. McDonald & Martin Kreitman

Department of Ecology and Evolutionary Biology, Princeton University, Princeton, New Jersey 08544, USA

**PROTEINS often differ in amino-acid sequence across species. This difference has evolved by the accumulation of neutral mutations by random drift, the fixation of adaptive mutations by selection, or a mixture of the two. Here we propose a simple statistical test of the neutral protein evolution hypothesis based on a comparison of the number of amino-acid replacement substitutions to synonymous substitutions in the coding region of a locus. If the observed substitutions are neutral, the ratio of replacement to synonymous fixed differences between species should be the same as the ratio of replacement to synonymous polymorphisms within species. DNA sequence data on the *Adh* locus (encoding alcohol dehydrogenase, EC 1.1.1.1) in three species in the *Drosophila melanogaster* species subgroup do not fit this expectation; instead, there are more fixed replacement differences between species than expected. We suggest that these excess replacement substitutions result from adaptive fixation of selectively advantageous mutations.**

Consider a set of alleles from more than one species and assume that there is no recombination. The alleles are then connected by a single phylogenetic tree, which can be divided into two parts: between-species branches and within-species branches. Within-species branches connect all the alleles within each species to their most recent common ancestor. Between-species branches connect these common ancestors to the common ancestor of the whole phylogeny. A mutation on a between-species branch will appear in all the descendant alleles and thus will be a fixed difference between species, whereas a mutation on a within-species branch will be a polymorphism within a species.

Nucleotide substitutions in a coding region can also be divided into replacement substitutions and synonymous substitutions. Of  $M$  possible mutations in a coding region, let  $M_r$  be the number of possible neutral replacement mutations and  $M_s$  be the number of possible neutral synonymous mutations. All remaining mutations,  $M - (M_r + M_s)$ , are deleterious. Let  $\mu$  be the nucleotide mutation rate per nucleotide site, so that the mutation rate for any one of the three possible mutations at a site is  $\mu/3$ . Under the neutral theory, the expected number of fixed replacement substitutions in a set of alleles is  $T_b(\mu/3)M_r$ , where  $T_b$  is the total time on the between-species branches. The expected number of fixed synonymous substitutions is

$T_b(\mu/3)M_s$ . For a particular phylogeny and mutation rate, the number of replacement substitutions is independent of the number of synonymous substitutions. Therefore, the expected ratio of replacement to synonymous fixed substitutions is  $T_b(\mu/3)M_r : T_b(\mu/3)M_s$ , which reduces to  $M_r : M_s$ . If  $T_w$  is the total time on the within-species branches of the phylogeny, the expected ratio of replacement to synonymous polymorphisms is  $T_w(\mu/3)M_r : T_w(\mu/3)M_s$ , which also reduces to  $M_r : M_s$ . Thus, if protein evolution occurs by neutral processes, the ratio of replacement to synonymous fixed substitutions should be the same as the ratio of replacement to synonymous polymorphisms. A  $G$ -test of independence<sup>1</sup> can be used to test this null hypothesis.

This test does not require many assumptions. Unlike most tests of the neutral theory, it does not require that populations have reached equilibrium. Recombination could cause different segments of a locus to have different phylogenies, but this would only cause a serious bias in the above test if the time to a common ancestor of a genomic segment was correlated with the ratio of neutral replacement to synonymous sites in that segment. There is no reason to expect such a correlation. Similarly, variation in nucleotide mutation rate among regions of a locus would only be important if nucleotide mutation rate was correlated with the ratio of neutral replacement to synonymous sites, which does not seem likely. We have assumed, for simplicity, that all nucleotide mutations are equally likely. A difference in mutation rate between transitions and transversions, coupled with the greater proportion of transitions that are synonymous, would change the ratio of replacement to synonymous substitutions. But the ratio for fixed substitutions would still equal the ratio for polymorphisms. If there are more synonymous sites than replacement sites with two or three possible neutral mutations, multiple substitutions at a single nucleotide site will make the ratio of replacement to synonymous fixed differences a slight overestimate of  $M_r : M_s$ . For closely related species the effect is trivially small and even in the most extreme case of complete saturation of neutral mutable sites with substitutions, the overestimation from multiple substitutions is not large enough to account for the results presented below.

We have compared DNA sequences of the coding region of the *Adh* locus in three species in the *Drosophila melanogaster* species subgroup (Table 1). There is substantial evidence that the threonine/lysine polymorphism in the alcohol dehydrogenase of *D. melanogaster* is subject to natural selection<sup>2,3</sup>, but there has been no evidence to indicate whether any of the replacement differences between species were adaptive. We limited the comparison to closely related species to minimize the number of multiple mutations at single nucleotide sites. The ratio of replacement substitutions to synonymous substitutions that are fixed between species is significantly greater than the ratio of replacement to synonymous polymorphisms (Table 2). About 29% of the fixed differences between species are replacement substitutions, but only 5% of the polymorphisms are replacement substitutions. This is not what would be expected if the observed replacement and synonymous substitutions were selectively neutral. It would be expected, however, if most of the observed replacement substitutions were due to adaptive fixation of selectively advantageous mutations. A substitution which becomes fixed by selection will be a polymorphism for less time than a substitution that becomes fixed by random drift<sup>4</sup> and so an adaptive substitution will be less likely to appear as a polymorphism than will a neutral substitution.

An alternative explanation for the excess number of fixed replacement differences could be a combination of many slightly deleterious replacement mutations and population sizes which have recently undergone dramatic expansion in all three species. In the ancestral small populations, mildly deleterious replacement mutations are effectively neutral and would therefore accumulate as fixed differences. In recently expanded populations, slightly deleterious replacements are selected against and



TABLE 1. Variable nucleotides from the coding region of the *Adh* locus in *D. melanogaster*, *D. simulans* and *D. yakuba*

|      | Con. | <i>D. melanogaster</i> |   |   |   |   |   |   |   |   |   |   |   | <i>D. simulans</i> |   |   |   |   |   | <i>D. yakuba</i> |   |   |   |   |   |   |   |   |   |   |   |       |         |       |       |         |       |
|------|------|------------------------|---|---|---|---|---|---|---|---|---|---|---|--------------------|---|---|---|---|---|------------------|---|---|---|---|---|---|---|---|---|---|---|-------|---------|-------|-------|---------|-------|
|      |      | a                      | b | c | d | e | f | g | h | i | j | k | l | a                  | b | c | d | e | f | a                | b | c | d | e | f | g | h | i | j | k | l |       |         |       |       |         |       |
| 781  | G    | T                      | T | T | T | T | T | T | T | T | T | T | T | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | Repl. | Fixed   |       |       |         |       |
| 789  | T    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | Syn.  | Fixed   |       |       |         |       |
| 808  | A    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | Repl. | Fixed   |       |       |         |       |
| 816  | G    | T                      | T | T | T | - | - | - | - | - | - | T | - | T                  | T | T | T | T | T | -                | - | - | - | - | - | - | - | - | - | - | - | -     | Syn.    | Poly. |       |         |       |
| 834  | T    | -                      | - | - | - | - | - | - | - | - | - | - | - | C                  | C | - | - | C | - | -                | - | - | - | - | - | - | - | - | - | - | - | Syn.  | Poly.   |       |       |         |       |
| 859  | C    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | Repl. | Fixed   |       |       |         |       |
| 867  | C    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | Syn.  | 2 Poly. |       |       |         |       |
| 870  | C    | T                      | T | T | T | T | T | T | T | T | T | T | T | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | Syn.    | Fixed |       |         |       |
| 950  | G    | -                      | - | - | - | - | - | - | - | - | - | - | - | A                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | Syn.    | Poly. |       |         |       |
| 974  | G    | -                      | - | - | - | - | - | - | - | - | - | - | - | T                  | - | T | T | T | T | -                | - | - | - | - | - | - | - | - | - | - | - | -     | Syn.    | Poly. |       |         |       |
| 983  | T    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | Syn.    | Fixed |       |         |       |
| 1019 | C    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | Syn.    | Poly. |       |         |       |
| 1031 | C    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | Syn.    | Poly. |       |         |       |
| 1034 | T    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | Syn.    | Poly. |       |         |       |
| 1043 | C    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | Syn.    | Poly. |       |         |       |
| 1068 | C    | T                      | T | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | Syn.  | Poly. |         |       |
| 1089 | C    | -                      | - | - | - | - | - | - | - | - | - | - | - | A                  | A | A | A | A | A | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | Repl. | Fixed |         |       |
| 1101 | G    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | Repl. | Fixed |         |       |
| 1127 | T    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | Syn.  | Fixed |         |       |
| 1131 | C    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | Syn.  | Poly. |         |       |
| 1160 | T    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | Syn.  | Fixed |         |       |
| 1175 | T    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | Syn.  | Fixed |         |       |
| 1178 | C    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | Syn.  | Poly. |         |       |
| 1184 | C    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | Syn.  | Fixed |         |       |
| 1190 | C    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | Syn.  | Fixed |         |       |
| 1196 | G    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | Syn.  | Poly. |         |       |
| 1199 | C    | -                      | T | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | Syn.  | Poly. |         |       |
| 1202 | T    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | Syn.  | Fixed |         |       |
| 1203 | C    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | Syn.  | Poly. |         |       |
| 1229 | T    | -                      | - | C | C | C | C | C | C | C | C | C | C | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | Syn.  | Poly. |         |       |
| 1232 | T    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | Syn.  | Fixed |         |       |
| 1235 | C    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | Syn.  | Poly. |         |       |
| 1244 | C    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | Syn.  | Poly. |         |       |
| 1265 | C    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | Syn.  | Fixed |         |       |
| 1271 | A    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | Syn.  | Poly. |         |       |
| 1277 | T    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | Syn.  | Fixed |         |       |
| 1283 | C    | A                      | A | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | Syn.  | Poly. |         |       |
| 1298 | C    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | Syn.  | Fixed |         |       |
| 1304 | C    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | Syn.  | Poly. |         |       |
| 1316 | C    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | Syn.  | Poly. |         |       |
| 1425 | C    | A                      | A | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | -     | Syn.  | Poly.   |       |
| 1431 | T    | C                      | C | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | -     | Syn.  | Poly.   |       |
| 1443 | C    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | -     | Syn.  | Poly.   |       |
| 1452 | C    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | -     | Syn.  | Poly.   |       |
| 1490 | A    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | -     | Repl. | Poly.   |       |
| 1504 | C    | T                      | T | T | T | T | T | T | T | T | T | T | T | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | -     | Syn.  | Fixed   |       |
| 1518 | C    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | -     | Syn.  | Poly.   |       |
| 1524 | T    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | -     | Syn.  | Fixed   |       |
| 1527 | C    | T                      | T | T | T | T | T | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | -     | Syn.  | Poly.   |       |
| 1530 | G    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | -     | Syn.  | Poly.   |       |
| 1545 | T    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | -     | Syn.  | Fixed   |       |
| 1548 | C    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | -     | Syn.  | Poly.   |       |
| 1551 | C    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | -     | Syn.  | Poly.   |       |
| 1555 | C    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | -     | Repl. | Poly.   |       |
| 1557 | C    | A                      | A | A | A | A | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | -     | Syn.  | Poly.   |       |
| 1560 | G    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | -     | Syn.  | Poly.   |       |
| 1573 | G    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | -     | Repl. | Fixed   |       |
| 1581 | C    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | -     | Syn.  | Fixed   |       |
| 1584 | C    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | -     | Syn.  | Poly.   |       |
| 1590 | C    | T                      | T | T | T | T | T | T | T | T | T | T | T | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | -     | -     | Syn.    | Poly. |
| 1596 | G    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | -     | -     | Syn.    | Poly. |
| 1611 | A    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | -     | Syn.  | Fixed   |       |
| 1614 | C    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - | - | - | - | -     | -       | -     | Syn.  | 2 Poly. |       |
| 1635 | C    | -                      | - | - | - | - | - | - | - | - | - | - | - | -                  | - | - | - | - | - | -                | - | - | - | - | - | - | - | - |   |   |   |       |         |       |       |         |       |

The first column contains the nucleotide positions, numbered according to ref. 6, so that the first base of the coding region of exon 2 is 778, of exon 3 is 942 and exon 4 is 1,417. The second column contains the consensus (con.) nucleotide for each site. Nucleotides identical to the consensus are shown as a dash. Several *D. yakuba* individuals were heterozygous; sites which are underlined contained both the nucleotide shown and the consensus nucleotide. The *D. melanogaster* sequences are six *Adh*<sup>s</sup> (a-f) and five *Adh*<sup>f</sup> (g-k) alleles<sup>6</sup> and one *Adh*<sup>FCD</sup> allele<sup>10</sup>. *D. simulans* sequences a and b were also from the literature.<sup>11,12</sup> *D. simulans* sequences c-f were of cloned alleles from flies from Australia, Canada, France and the Congo. They were sequenced by the dideoxy chain termination method directly from supercoiled plasmids<sup>13</sup>. The *D. yakuba* alleles of single flies from Brazzaville, Congo were sequenced directly from products of amplification by the polymerase chain reaction<sup>14,15</sup>. Each of these sequences is from one fly from a separate isofemale line. Every allele was completely sequenced in both directions. Each substitution relative to the consensus nucleotide at a site is classified as either fixed or polymorphic. A substitution that is fixed in one species and polymorphic in another is classified as a polymorphism. Because we do not know whether a substitution that is polymorphic in more than one species represents one or two mutations, it is classified as a single polymorphism. Syn, synonymous; Repl, replacement; Poly, polymorphic.

would not appear as polymorphisms. This explanation requires that many replacement mutations have selection coefficients against them that make them neutral in each small population but selected against in each expanded population. It also requires that each population has been large for long enough that slightly deleterious polymorphisms have been eliminated by selection, but not for so long that slightly advantageous back-mutations have replaced the slightly deleterious fixed substitutions. This slightly deleterious model requires so many assumptions about selection coefficients, population sizes, and the times of population expansions that we prefer the simpler explanation, the occasional fixation of an adaptive mutation.

The rate of neutral synonymous mutation at the *Adh* locus in *Drosophila* has been estimated to be about  $8 \times 10^{-9}$  per synonymous site per year<sup>5</sup>. There are about 192 effectively synonymous sites in the coding regions of the *Adh* locus<sup>6</sup>, so that the sum of the lengths of the between-species branches of the phylogeny connecting *D. melanogaster*, *D. simulans* and *D. yakuba*, with 17 synonymous substitutions, is about 11 million years (ignoring multiple hits for this rough calculation). If all seven fixed replacement substitutions were adaptive, there was an average of one adaptive fixation every 1.6 million years. It has been suggested that for a plausible value of genetic load, one mutation could be fixed by selection every 300 generations<sup>7</sup>.

TABLE 2 Number of replacement and synonymous substitutions for fixed differences between species and polymorphisms within species

|             | Fixed | Polymorphic |
|-------------|-------|-------------|
| Replacement | 7     | 2           |
| Synonymous  | 17    | 42          |

A G-test of independence (with the Williams correction for continuity)<sup>1</sup> was used to test the null hypothesis, that the proportion of replacement substitutions is independent of whether the substitutions are fixed or polymorphic.  $G = 7.43$ ,  $P = 0.006$ .

More realistic models of selection suggest that adaptation could occur much more quickly<sup>8,9</sup>, but if we accept the conservative figure and assume there are 10 generations per year in *Drosophila*, it would mean that 50,000 loci could be undergoing adaptive evolution as quickly as *Adh*. Thus, the rate of adaptive protein evolution seen at the *Adh* locus may not be unusually

high, and selective fixation of adaptive mutations may be a viable alternative to the clocklike accumulation of neutral mutations as an explanation for most protein evolution. □

Received 20 March; accepted 13 May 1991.

1. Sokal, R. R. & Rohlf, F. J. *Biometry* (Freeman, San Francisco, 1981).
2. Lewontin, R. C. *A. Rev. Genet.* **19**, 81–102 (1985).
3. Kreitman, M. & Hudson, R. R. *Genetics* **127**, 565–582 (1991).
4. Nei, M. *Molecular Evolutionary Genetics* (Columbia University Press, New York, 1987).
5. Sharp, P. M. & Li, W.-H. *J. molec. Evol.* **28**, 398–402 (1989).
6. Kreitman, M. *Nature* **304**, 412–417 (1983).
7. Haldane, J. B. S. *J. Genet.* **57**, 511–524 (1957).
8. Maynard Smith, J. *Nature* **219**, 1114–1116 (1968).
9. Sved, J. A. *Am. Nat.* **102**, 283–293 (1968).
10. Collet, C. *J. molec. Evol.* **27**, 142–146 (1988).
11. Bodmer, M. & Ashburner, M. *Nature* **309**, 425–430 (1984).
12. Cohn, V. H. & Moore, G. P. *Molec. Biol. Evol.* **5**, 154–166 (1988).
13. Zagursky, R. J., Baumeister, K., Lomas, N. & Berman, M. L. *Gene Analyt. Techn.* **2**, 89–94 (1985).
14. Saiki, R. K., Bugawan, T. L., Horn, G. T., Mullis, K. B. & Erlich, H. A. *Nature* **324**, 163–166 (1988).
15. Higuchi, R. G. & Ochman, H. *Nucleic Acids Res.* **17**, 5865 (1989).

ACKNOWLEDGEMENTS. We thank S. El-Gogary for cloning the *D. simulans* alleles and J. R. David for the isofemale lines of *D. yakuba*. This work was supported by the NIH and the NSF. DNA sequences have been deposited in GenBank, accession numbers X57361–X57364 (*D. simulans*) and X57365–X57376 (*D. yakuba*).

## Development of the light response in neonatal mammalian rods

G. M. Ratto, D. W. Robinson, B. Yan\* & P. A. McNaughton\*

Physiological Laboratory, Downing Street, Cambridge CB2 3EG, UK

THE sensitivity to light is low in many neonatal mammals when compared with that in the adult. In human infants at one month of age, for example, the dark-adapted sensitivity for detection of large stimuli is 50 times lower than in the adult<sup>1–4</sup>, and in rats the overall sensitivity of the neonatal retina is also low compared with the adult<sup>5,6</sup>. This low sensitivity in the neonate has been attributed to a number of factors<sup>5–11</sup>, but the possibility that the photoreceptors themselves might be an important limitation on the overall visual sensitivity has not so far been clearly established. Here we record the light response of single neonatal rat rods and find that the sensitivity is considerably lower than in the adult. The response to a single photoisomerization is normal in the neonate, and the sensitivity deficit can therefore be attributed to a low level of functional rhodopsin. Opsin, the protein component of rhodopsin, must be present in normal amounts, as the sensitivity can be restored to adult levels by treating the retina with 9-*cis* retinal, an active homologue of the native chromophore 11-*cis* retinal. The low sensitivity of photoreceptors in the neonate can therefore be attributed mainly to a low concentration of 11-*cis* retinal in the developing retina.

Rod outer segments begin to appear around the optic disk at about postnatal day 8 (P8) in the rat, and they grow rapidly towards the adult size over the next three weeks<sup>12</sup>. We have recorded the membrane current of single rod outer segments, using the suction pipette method<sup>13</sup>, from animals ranging in age from P12 (the earliest time at which any response to light has been detected) to adult (age greater than two months). Figure 1 compares typical recordings obtained from rods from a one-month-old animal and a 13-day-old animal. There are two principal respects in which the responses from neonatal rods differ from those in more mature animals: the light-sensitive current is smaller, and the sensitivity to light is less by more than an

order of magnitude. As a qualitative illustration of the second point, the flash strength giving the smallest response in the P13 rod shown in Fig. 1b is the same as that giving the second-largest response in the P35 rod in Fig. 1a.

The intensity-response relations for rods from albino rats of various ages are shown in Fig. 2. In each case the points have been fitted by an exponential function<sup>14</sup> (see legend to Fig. 2), which provides a good fit to the intensity-response relation of other mammalian rods<sup>15</sup>. The sensitivity is inversely proportional to the light intensity required to produce a half-saturating response. Only a small fraction of rods at P13 responded to light and, of those that did, the sensitivity was on average 49 times less than in the adult. Rods from 17-day-old animals are seven times less sensitive than those from adults, and at P35, when the animals have been weaned and the outer segments are almost of adult size, the sensitivity is still three times less than in the adult. Similar results were obtained from pigmented rats (see Fig. 2 legend).

There are several mechanisms which might account for the low sensitivity of the neonate. Some components of the light-sensitive pathway may not be fully expressed, resulting in a reduced response to a single photoisomerization. A second possibility is that fewer photons of light may be absorbed in the neonatal outer segments, either because of a delay in expression of the gene coding for opsin, or because an insufficient supply of the light-sensitive chromophore 11-*cis* retinal is available. The outer segment length at P13 is one half of adult (see Fig. 2 legend), but this difference does not account for the low sensitivity (see Fig. 3 legend). The possibility that the action spectrum of neonatal rhodopsin might be different can also be discarded<sup>16</sup>.

The response to a single photoisomerization has been determined in the experiments shown in Fig. 3 by observing the responses to a series of dim flashes<sup>15,17</sup>. The single-photon response in this adult (Fig. 3, top) was 0.2 pA, or 4.2% of the maximum response, whereas in the P13 rod (Fig. 3, bottom), which was 56 times less sensitive, the single photon response was 0.42 pA, or 21% of the saturating response. In four experiments on animals aged between P13 and P17 the mean single-photon response was 0.58 pA, and in seven experiments on animals P30 or older, the mean response was 0.50 pA (for further details see legend to Fig. 3). The gain of the light-sensitive pathway is therefore not significantly different in the neonate, and the low sensitivity must be due to a reduced absorption of light in the immature outer segments.

If there is a delay in the expression of the gene coding for opsin, then the absorption of light should be localized to the

\* Present addresses: Department of Microbiology, University of Texas Medical School at Houston, PO Box 20708, Houston, Texas 77225, USA (B.Y.); and Physiology Subject Group, Department of Biomedical Sciences, King's College London, Strand, London WC2R 2LS, UK (P.A.McN.).



FIG. 1 Responses of outer segment membrane current, recorded from rods from a 35-day-old rat (*a*) and a 13-day-old rat (*b*) to flashes of 495 nm light (timing of 20-ms flash given by flash monitor below traces). The outer segment current of single rods in small fragments of retina was recorded using the suction electrode method<sup>13,15</sup>. Outer segments were illuminated transversely with diffuse unpolarized light. Flash intensities (in photons per  $\mu\text{m}^2$ ) were, from bottom: *a*, 55, 210, 300, 450, 1,150 and 7,600; *b*, 1,140, 2,300, 6,300, 14,000, 44,000. Traces with similar flash intensities are identified with a diamond symbol. Half-saturating light intensity, determined as in Fig. 2, was 303 photons per  $\mu\text{m}^2$  for the P35 rod, and 2,988 photons per  $\mu\text{m}^2$  for the P13 rod. Temperature was 38.8 °C in *a*, and 39 °C in *b*. Data were smoothed digitally by an 11-point averaging window of duration 25 ms. Traces are the average of between 16 presentations (for bright-flash responses) and 50 presentations (for dim-flash responses). Similar responses to those shown for the P35 animal were recorded from adult rods, but the adults were on average 3 times more sensitive (see Figs 2 and 4).

**METHODS.** Rats (albino CHFB strain or pigmented Lister Hooded strain) were fed on rat and mouse diet number 3 (Special Diet Services, Witham, Essex) containing 11.1 mg kg<sup>-1</sup> vitamin A (36,600 i.u. per kg), and were maintained in a cycle of 12 h light (mean intensity at the cages 7 lm m<sup>-2</sup>), 12 h dark. Animals were dark-adapted for at least 1 h, killed under dim red light by CO<sub>2</sub> anaesthesia followed by cervical dislocation, and the retina rapidly isolated under infrared illumination. The central portion of the retina, which is where development is most advanced in the young animal, was excised under infrared light and finely chopped in a modified Hank's solution (in mM: NaCl, 130; KCl, 5.4; MgCl<sub>2</sub>, 0.5; MgSO<sub>4</sub>, 0.2; CaCl<sub>2</sub>, 1; ascorbic acid, 2; glucose, 5; HEPES, 10, pH 7.5). Retinae were either used immediately or stored for 1–8 h in Hank's with 10 mM glucose at 5 °C before use. Perfusion solution was warmed to 39 °C by a Peltier device controlled by the feedback circuit. Bath temperature was continuously monitored by a miniature thermocouple. Current was low-pass filtered to 30 Hz by a 6-pole filter and was digitized at 400 Hz using a CED 1401 interface to an IBM PC-AT computer. Methods otherwise as described in ref. 23.

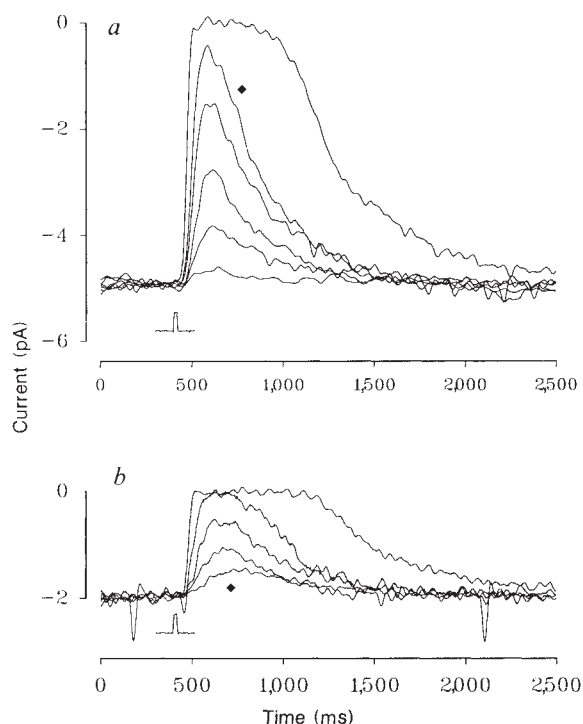
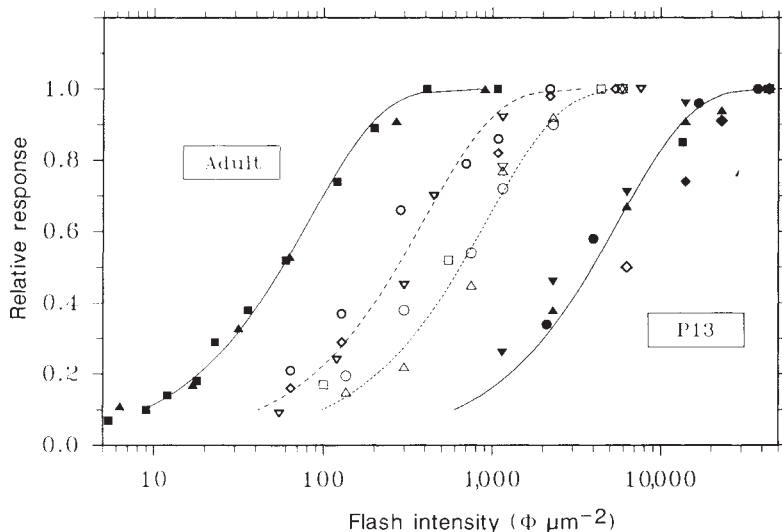


FIG. 2 Representative examples of intensity-response relations obtained from rods from albino and pigmented rats. Response relative to maximum (ordinate) is plotted as a function of flash intensity in photons per  $\mu\text{m}^2$  (abscissa). Points show results from one rod from an adult albino (filled triangles to left) and one from an adult pigmented rat (filled squares to left); three rods from rats of mean age P35 (range P30–P40; bold open symbols); four rods from P17 rats (light open symbols); and six rods from three 13-day rats (filled symbols and bold diamond to right). Continuous curves show relation  $R = 1 - e^{-KI}$  (ref. 14), where  $R$  is the response relative to maximum,  $I$  is the flash intensity and  $K$  is a constant. The position of the curves on the abscissa is most readily characterized by the value of light intensity required to produce a half-saturating response, which is inversely proportional to the sensitivity. Curves have been fitted to the data shown here; means and standard deviations of the half-saturating light intensities, for the various populations of rods, are shown as the open symbols in Fig. 4. New outer segments continue to emerge as the retina develops, but for the purposes of this study all measurements of light response and outer segment length were taken from rods that are the first to appear and are therefore the longest. Mean light-sensitive current ( $\pm$ s.e.m.) of those rods whose sensitivity was determined in the present study was: P13,  $1.43 \pm 0.17$  pA; P17,  $2.69 \pm 0.33$  pA; P35,  $4.57 \pm 0.44$  pA; adult,  $5.27 \pm 0.79$  pA. Outer segment lengths for some of the rods from which full  $I-R$  relations had been obtained were determined directly from the television monitor during the experiment, and mean lengths were: P13,  $12.5 \pm 0.85$   $\mu\text{m}$ ; P35,  $21.8 \pm 0.6$   $\mu\text{m}$ ; adult,  $25.3 \pm 2.9$   $\mu\text{m}$ . The development of light-sensitive current therefore lags behind the growth of outer-segment length, with a current 27% of the adult being recorded at P13, when outer segments have attained 50% of their adult length. Diameters of rod outer segments were determined from living retinal tissue using Nomarski optics in conditions similar to those of the experiment. Diameter increased slightly with age, with values (mean  $\pm$  s.e.m.) of  $1.39 \pm 0.07$   $\mu\text{m}$  ( $n=12$ ),  $1.47 \pm 0.03$   $\mu\text{m}$  ( $n=59$ ) and  $1.52 \pm 0.05$   $\mu\text{m}$  ( $n=21$ ) being measured in the 9-day, 13-day and adult animals, respectively. The increase



in diameter after P13 is therefore less than 10% (95% confidence limit). Electron micrographs of neonatal outer segments showed that the ultrastructure was substantially normal, with discs fully formed and separated from the surface membrane as in adult rod outer segments. The possibility that the sensitivity change may be related to the retinal degeneration that occurs in adult albino rats<sup>24</sup> has been investigated by recording from pigmented rats (Lister Hooded strain). The form and sensitivity of the light responses in adult pigmented rats were found to be indistinguishable from those in albino adults (filled squares in Fig. 2). The sensitivity increase during development is also present in pigmented rats, although it occurs later in these animals; rods at P22 are 29 times less sensitive than in the adult ( $n=5$ ).

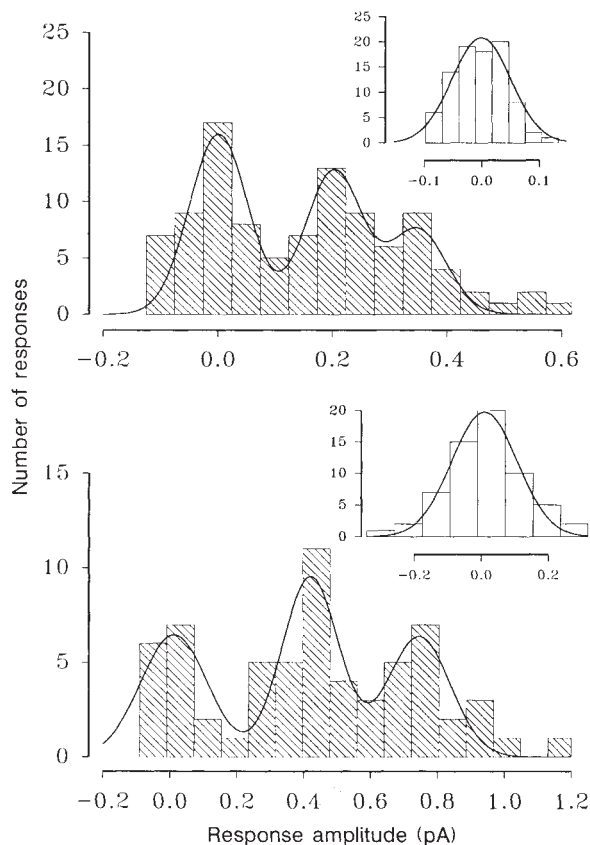


FIG. 3 Amplitude histogram for dim-flash responses recorded from an adult rod (top) and a rod from a P13 animal (bottom). The light-sensitive current was recorded as in Fig. 1 and a series of dim flashes delivered ( $n=103$  for the adult, intensity 5.4 photons per  $\mu\text{m}^2$ ; and  $n=63$  for the P13 rod, intensity 1,140 photons per  $\mu\text{m}^2$ ). Peak amplitude of the response to each flash was measured by calculating the average response for the entire series and least-squares fitting this ensemble average to each individual response. The insets show amplitude histograms obtained using an identical procedure on interleaved traces in which no flash was delivered. Half-saturating light intensity, determined as shown in Fig. 2, was 53 photons per  $\mu\text{m}^2$  for this adult rod and 2,988 photons per  $\mu\text{m}^2$  for the P13 rod, and dark current was 4.7 pA and 2.0 pA, respectively. Continuous curves in the insets show a single gaussian function best-fitted to the 'blank' histograms using a Levenberg-Marquardt minimization algorithm (SigmaPlot, Jandel Corp.). The sum of three gaussian functions was then best-fitted to the 'flash' histograms with the mean and  $\sigma$  of the first constrained to be the same as was obtained for the blank trials (compare ref. 15). Means of gaussians give the peak current change produced by zero, one and two photoisomerizations, and had values  $-0.01$  pA, 0.20 pA and 0.35 pA for the adult, and 0.01 pA, 0.42 pA and 0.75 pA for P13. The single-photon event was therefore 0.2 pA, or 4.2% of the maximum response, in this adult and 0.42 pA, or 21% of the maximum, in the P13 rod. As a check, the areas of the three gaussian curves, which are proportional to the probabilities of 0, 1 and 2 photoisomerizations, were compared with the probabilities predicted by the Poisson distribution, using the observed mean event frequency. Observed probabilities, with the predicted values in parentheses, were for the adult,  $P(0)=0.45$  (0.47);  $P(1)=0.35$  (0.36);  $P(2 \text{ or more})=0.20$  (0.17); for the P13,  $P(0)=0.32$  (0.38);  $P(1)=0.39$  (0.37);  $P(2 \text{ or more})=0.29$  (0.25). In 7 experiments on rods from animals older than P30, the single-photon event was  $0.50 \pm 0.08$  pA (mean  $\pm$  s.e.m.), or  $13.9 \pm 2.4\%$  of the maximum, and in 4 experiments on animals between P13 and P17, the single-photon event was  $0.58 \pm 0.16$  pA, or  $23.7 \pm 2.8\%$  of the maximum. The gain of the light-sensitive pathway is therefore similar in adults and neonates, when expressed in terms of the absolute response to a single photoisomerization. When expressed in terms of fraction of the light-sensitive current suppressed by a single photoisomerization, the gain is about double in the neonate, but when account is taken of the twofold difference in length the sensitivity, expressed in terms of photons per  $\mu\text{m}^2$  required for half-saturation, would be similar if the densities of rhodopsin were equal.

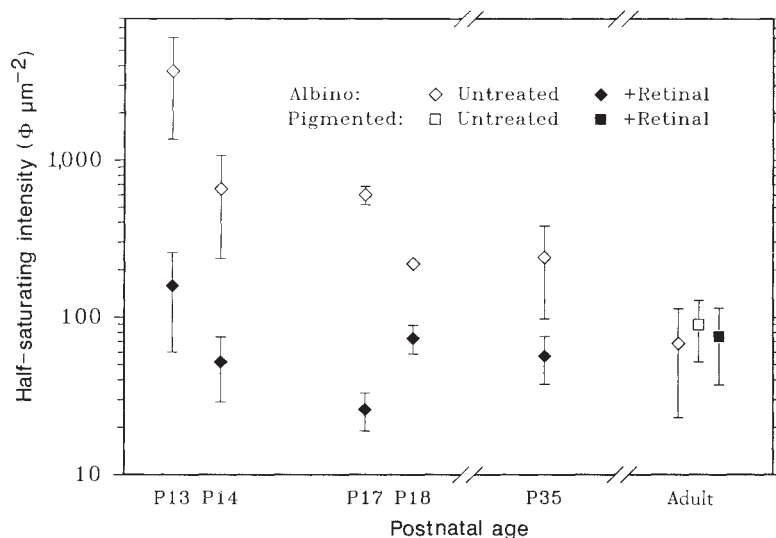
base of the outer segment, as the outer segments grow continuously from the base and opsin is not believed to be capable of diffusing between discs along the length of the outer segment<sup>18</sup>. We have not found any striking difference in sensitivity in experiments where the base and tip of neonatal outer segments were illuminated with transversely oriented slits of light (compare ref. 19), and active rhodopsin appears therefore to be distributed along the length of the outer segment.

If opsin is present in normal quantities, then it should be possible to regenerate the adult sensitivity in neonatal retinæ

by the addition of the native light-sensitive chromophore 11-*cis*-retinal. We have used instead the analogue 9-*cis*-retinal, which is almost equally effective<sup>20</sup> and is commercially available. Rods from neonatal retinæ treated with 9-*cis*-retinal exhibit a dramatically increased sensitivity; at P13 the sensitivity of the treated rods is more than ten times greater than in untreated rods, and from P14 the treated cells exhibit a sensitivity comparable to the adult (Fig. 4).

The low sensitivity of neonatal rat rods can therefore be attributed to an inadequate supply of 11-*cis* retinal in the

FIG. 4 Development of light sensitivity as a function of postnatal age in rods from untreated retinæ (open symbols) and in rods from retinæ pretreated for 2–4 h at 4 °C with Hank's solution containing 10 nM 9-*cis*-retinal (Aldrich; diluted from a 35  $\mu\text{M}$  ethanolic stock; ethanol in the concentrations used was without effect). Bars show the standard deviation about the points. Numbers of experiments were: P13, untreated 8, treated 5; P14, 2, 3; P17, 4, 2; P18, 1, 4; P35, 9, 6; adult albino, 4, 0; adult pigmented, 6, 9.  $\Phi$ , Photons.





developing retina, and not to a deficit in other components of the light-sensitive pathway. If a similar mechanism operates in human infants then the sensitivities of both rod and cone vision should be reduced, as both types of photoreceptor depend on a supply of 11-*cis* retinal for the regeneration of light-sensitive pigment. This reduction in sensitivity would reduce visual acuity at all but the highest light levels. It is possible that defects in the development of the system supplying 11-*cis* retinal could thereby cause defects at higher levels of the visual system, which

depend on a high-quality image for their normal development (see ref. 21 for a review).

Retinoids are thought to be an important morphological signal in development (see, for example, ref. 22). One possible explanation for the low sensitivity of rods in the neonate is that the level of 11-*cis* retinal must be kept low during development and can only be allowed to rise to levels adequate for complete regeneration of light-sensitive pigment once the retina is essentially mature. □

Received 1 February; accepted 3 May 1991.

- Lewis, J. M. & Haig, C. *J. Pediatr.* **15**, 812-823 (1939).
- Powers, M. K., Schneek, M. & Teller, D. Y. *Vision Res.* **21**, 1005-1016 (1981).
- Hamer, R. D. & Schneek, M. *Vision Res.* **24**, 77-85 (1984).
- Fulton, A. B., Hansen, R. M., Yeh, Y.-L. & Tyler, C. W. *Vision Res.* **31**, 1259-1269 (1991).
- Fulton, A. B. & Graves, A. L. *Vision Res.* **20**, 819-826 (1980).
- Fulton, A. B. & Baker, B. N. *Invest. Ophthalmol. Vis. Sci.* **25**, 647-651 (1984).
- Brown, A. M. *Vision Res.* **26**, 707-710 (1986).
- Carter-Dawson, L. et al. *Dev. Biol.* **116**, 431-438 (1986).
- Wilson, H. R. *Vision Res.* **28**, 611-628 (1988).
- Jacobs, D. S. & Blakemore, C. B. *Vision Res.* **28**, 947-958 (1988).
- Banks, M. S. & Bennett, P. J. *J. Opt. Soc. Am. A* **5**, 2059-2079 (1988).
- Dowling, J. E. & Sidman, R. L. *J. Cell Biol.* **14**, 73-105 (1961).
- Baylor, D. A., Lamb, T. D. & Yau, K.-W. *J. Physiol.* **288**, 589-611 (1979).
- Lamb, T. D., McNaughton, P. A. & Yau, K.-W. *J. Physiol.* **319**, 463-496 (1981).

- Baylor, D. A., Nunn, B. J. & Schnapf, J. L. *J. Physiol.* **357**, 575-607 (1984).
- Alpern, M., Fulton, A. B. & Baker, B. N. *Vision Res.* **27**, 1459-1470 (1987).
- Baylor, D. A., Lamb, T. D. & Yau, K.-W. *J. Physiol.* **288**, 613-634 (1979).
- Young, R. W. *Invest. Ophthalmol. Vis. Sci.* **15**, 700-725 (1976).
- McNaughton, P. A., Yau, K.-W. & Lamb, T. D. *Nature* **283**, 85-87 (1980).
- Makino, C. L. et al. *J. Physiol.* **424**, 545-560 (1990).
- Wiesel, T. N. *Nature* **299**, 583-591 (1982).
- Brookes, J. P. *Neuron* **2**, 1285-1294 (1989).
- Hodgkin, A. L., McNaughton, P. A. & Nunn, B. J. *J. Physiol.* **358**, 447-468 (1985).
- Noell, W. K. *Vision Res.* **20**, 1163-1171, 1980.

ACKNOWLEDGEMENTS. We thank L. Lagnado for help in developing the preparation and the recording technique, M. Lunn for his participation in early experiments, and L. Cervetto, D. Burr and R. Perry for discussion. Supported by the European Community Stimulation Action Programme, the Medical Research Council and NATO.

## Reversible uncoupling of inactivation in N-type calcium channels

Mark R. Plummer\* & Peter Hess†

Department of Cellular and Molecular Physiology,  
Program in Neuroscience, Harvard Medical School,  
Boston, Massachusetts 02115, USA

**N-TYPE calcium channels are thought to be expressed specifically in neuronal cells<sup>1-3</sup> and to have a dominant role in the control of neurotransmitter release from sympathetic neurons<sup>4,5</sup>. But their unitary properties are poorly understood and the separation of neuronal  $\text{Ca}^{2+}$  current into components carried by N-type or L-type  $\text{Ca}^{2+}$  channels is controversial<sup>6</sup>. Here we show that individual N-type  $\text{Ca}^{2+}$  channels in sympathetic neurons can carry two kinetically distinct components of current, one that is rapidly transient and one that is long lasting. The mechanism that gives rise to these two components is unexpected for  $\text{Ca}^{2+}$  channels: a test depolarization elicits either a rapidly inactivating, single short burst with an average duration of 40 ms, or sustained, non-inactivating channel activity lasting for over 1 s. The switching between inactivating and noninactivating activity is a slow process, the occurrence of each type of unitary kinetic behaviour remaining statistically correlated over several seconds. Variable coupling of inactivation in N-type  $\text{Ca}^{2+}$  channels could be an effective mechanism for the modulation of neuronal excitability and synaptic plasticity.**

A widely used criterion to distinguish neuronal N-type  $\text{Ca}^{2+}$  channels from L-type  $\text{Ca}^{2+}$  channels sensitive to dihydropyridine (DHP) has been the assignment of inactivating (transient) components of high-threshold-activated inward  $\text{Ca}^{2+}$  current to N-type channels and of maintained current components to L-type channels<sup>7,8</sup>. But this distinction is problematic because the extent and rate of inactivation varies considerably among N-type channels in various neuronal cells<sup>2,7-14</sup>. A maximally effective concentration (10  $\mu\text{M}$ ) of  $\omega$ -conotoxin ( $\omega$ -CgTx), a selective

blocker of N-type  $\text{Ca}^{2+}$  channels in peripheral neurons<sup>2,3,9,15</sup>, blocks inactivating and noninactivating components of whole-cell  $\text{Ba}^{2+}$  current in a sympathetic neuron from a rat superior cervical ganglion (SCG neuron; Fig. 1). This result, consistent with previous reports of  $\omega$ -CgTx block in sympathetic neurons<sup>2,4</sup>, has suggested<sup>16</sup> that at least two  $\omega$ -CgTx-sensitive but kinetically distinct types of  $\text{Ca}^{2+}$  channels may exist, a rapidly inactivating one and a noninactivating one. But our analysis of single N-type channel activity reveals that the same N-type channel can give rise both to inactivating and to noninactivating behaviour. A series of depolarizing voltage steps produces two distinct patterns of gating (Fig. 2a): in some sweeps, channel activity occurs as a single short burst, immediately after the onset of the voltage-clamp depolarization. In other sweeps, the channel continues to show bursting activity for the duration of the 1.4-s depolarization. Both types of unitary activity are due to openings of the same channel, as no overlapping events were detected (see legend to Fig. 2). As expected, amplitude histograms from long-lasting and short bursts show identical open channel peaks (Fig. 2c). Sweeps were further divided into two separate groups: short (no openings detected later than 450 ms after the depolarization) and long lasting. Open-time distributions and cumulative latencies to first opening for short and long-lasting activity were nearly identical (Fig. 2e, f). Burst distributions revealed a single exponential component of bursts in sweeps with short activity, with a mean duration of 40 ms. By contrast, in 80% of sweeps with long-lasting activity, openings occurred until the time of repolarization (1.4 s), so that an average burst-length could not be determined this way. Thus the main difference between short- and long-lasting activity is the presence or absence of a transition into an inactivated state. Average currents obtained from each set of sweeps (Fig. 2b) illustrate the marked differences between the two kinetic patterns. The average of all single-channel traces contains the decaying and maintained current components of the  $\omega$ -CgTx-sensitive whole-cell  $\text{Ba}^{2+}$  current.

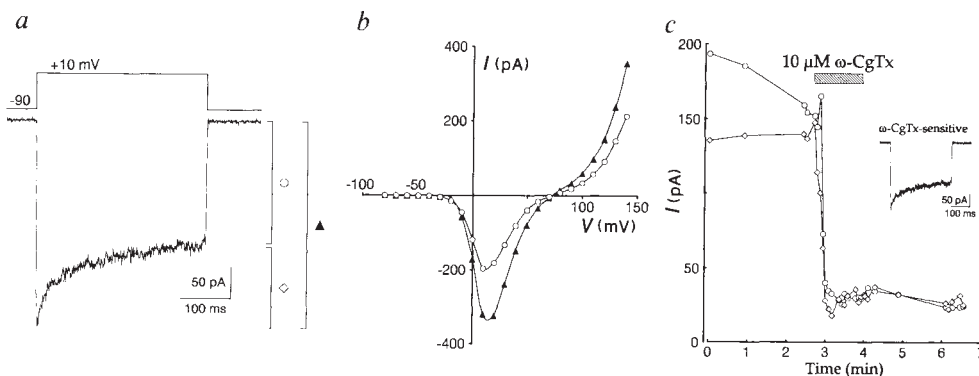
The process that reversibly switches a single N-type channel from inactivating to noninactivating is much slower than the rapid transitions between individual open and closed states. Figure 3 shows the sequences of the gating pattern observed in three different patches, each containing a single N-type channel. Channel activity elicited by individual depolarizations was classified either as nulls (no detectable channel openings), short

\* Present address: Department of Biological Sciences, Nelson Biological Labs, PO Box 1059, Rutgers University, Piscataway, New Jersey 08855-1059, USA.

† To whom correspondence should be addressed.

FIG. 1 Whole-cell current carried by N-type  $\text{Ca}^{2+}$  channels in an intact rat SCG neuron has an inactivating and a maintained component. *a*, Inward  $\text{Ba}^{2+}$  current elicited by a 340 ms depolarization from  $-90$  mV to  $+10$  mV. The charge carrier is  $20$  mM  $\text{Ba}^{2+}$ . *b*, Current-voltage relationship of the peak inward current ( $\blacktriangle$ ) and the maintained current ( $\circ$ ) for the same SCG neuron as in *a*. Both current components activate at positive potentials and are thus carried by high-threshold-activated channels. *c*,  $10$   $\mu\text{M}$   $\omega$ -CgTx irreversibly blocks both inactivating ( $\blacklozenge$ ) and maintained ( $\circ$ ) components. The  $\omega$ -CgTx (Peninsula Laboratories) was applied by a blunt micropipette during the time indicated (hatched bar). The  $\omega$ -CgTx-sensitive current (control current minus current after  $\omega$ -CgTx application) is shown in the inset. The same SCG neuron as in *a* and *b* is used. Holding potential  $-90$  mV, test potential  $+10$  mV.

METHODS. The techniques for cell isolation and patch-clamp recording were as previously described<sup>2</sup>. SCG neurons from neonatal rats were studied  $<24$  h after dissociation. Whole-cell recordings were obtained with the

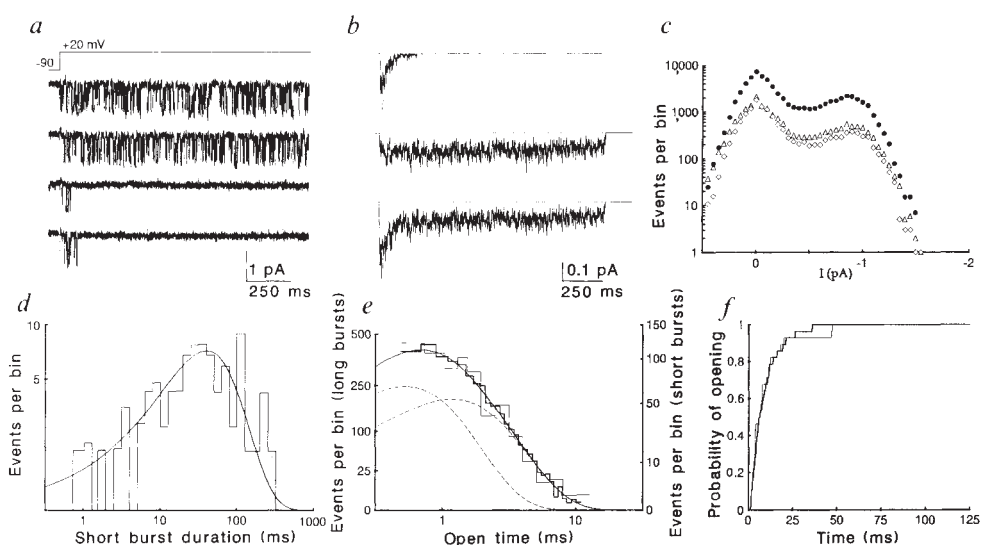


following solutions: bath solution,  $20$  mM barium acetate,  $135$  mM tetraethylammonium-aspartate,  $10$  mM HEPES, pH  $7.5$ ; pipette solution,  $145$  mM caesium-aspartate,  $10$  mM BAPTA ( $1,2$ -Bis(*o*-amino phenoxy)ethene- $N,N',N'$ -tetraacetic acid),  $4$  mM ATP,  $5$  mM  $\text{MgCl}_2$ ,  $10$  mM HEPES, pH  $7.5$ . All experiments were done at room temperature ( $22^\circ\text{C}$ ). Currents were digitized at  $500$   $\mu\text{s}$  per point and filtered at  $1$  kHz ( $-3\text{dB}$ , eight-pole Bessel filter). Linear leak and capacitance currents were subtracted digitally.

bursts or long bursts. We tested for statistical correlation between patterns of gating in consecutive sweeps recorded at  $5$ -s intervals. Pairs of consecutive sweeps were classified according to their gating pattern into the four possible combinations (short-short, short-long, long-short, long-long; Fig. 3*b*). The probability that the observed contingency tables arose from random association of short or long bursts, given the known overall occurrence of

each gating pattern during the entire experiment, was less than  $0.05$  for each of the patches, as assessed either by a  $\chi^2$  test<sup>17</sup>, Fisher's exact test<sup>17</sup> or by 'runs' analysis<sup>18,19</sup>. Thus, long- and short-bursting patterns remain correlated over the duration of the interpulse interval, and we estimate the average lifetimes of nulls, inactivating and noninactivating patterns as  $11.9$ ,  $5.1$  and  $6.2$  s, respectively (see legend to Fig. 3). The slow transition

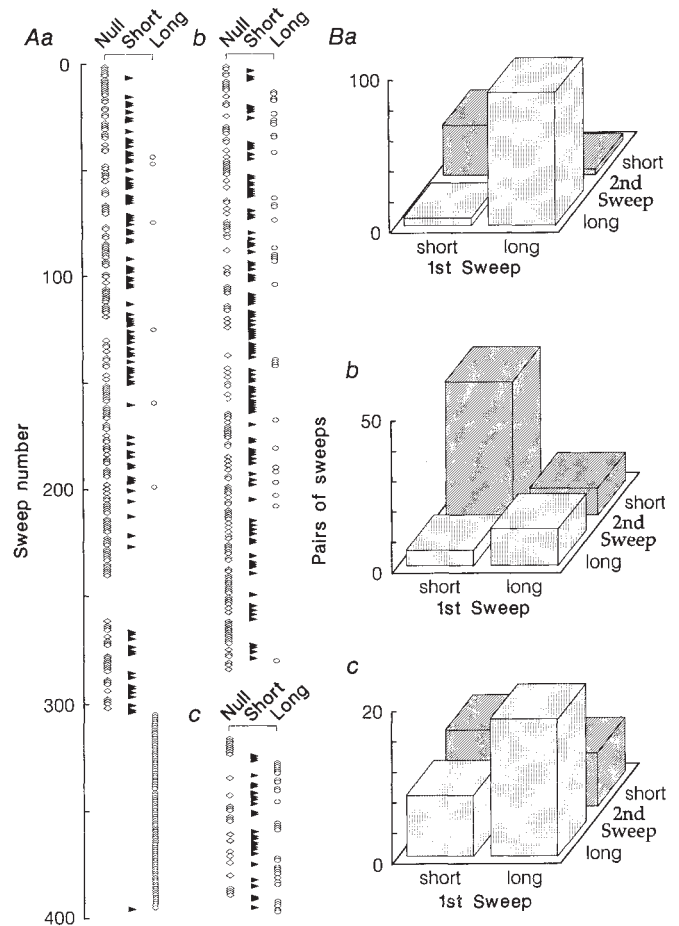
FIG. 2 Unitary activity of a single N-type channel. *a*, Cell-attached recording from a patch containing a single N-type channel. Selected traces (nonconsecutive) in response to  $1.4$ -s-long depolarizations from  $-90$  mV to  $+20$  mV with  $110$  mM  $\text{Ba}^{2+}$  as charge carrier. N-type  $\text{Ca}^{2+}$  channels were identified as previously reported<sup>2</sup>. The DHP agonist, (+)- $(S)$ -202-791 (gift of Sandoz, Basle, Switzerland), was present at  $500$  nM in the bath solution. Thus DHP-sensitive L-type  $\text{Ca}^{2+}$  channels could be identified<sup>2</sup> and patches containing L-type channels excluded from the analysis. The drug had no effect on the inactivation of whole-cell or single-channel N-type current in SCG neurons (ref. 2, and unpublished data). *b*, Averages from sweeps with short bursts (top,  $n=103$ ), long bursts (middle,  $n=28$ ) and all sweeps with channel activity (bottom trace,  $n=131$ ). Sweeps were classified as short bursts if no openings were detected later than  $450$  ms after the depolarizing voltage clamp step. *c*, Amplitude histograms from all sweeps during the first  $80$  ms of the pulse ( $\Delta$ ), all sweeps between  $0.5$  and  $1.3$  s after clamp depolarization ( $\bullet$ ) and of the short bursts only ( $\diamond$ ). The histogram is shown on a log scale to emphasize that no events attributable to simultaneous openings of more than one channel were detected. The peak at  $0$  pA represents the closed channel, the open channel peak is at  $-0.85$  pA and is the same for short and long bursts. *d*, Distribution of bursts from sweeps classified as short bursts, displayed as a square-root log plot<sup>26</sup>. The smooth line is the best fit (maximum likelihood estimate) to a single exponential distribution with a mean burst duration of  $40$  ms. The time ( $450$  ms) chosen to classify current traces as short bursts is very long compared to the mean burst length and thus should not bias the observed distribution. *e*, Superim-



posed open-time distributions from long bursts (bold trace, left-hand scale) and short bursts (right-hand scale). Smooth solid curve, best fitting sum of two exponential distributions (maximum likelihood estimate) with time constants of  $0.5$  and  $1.2$  ms, respectively. The individual exponential components are shown as broken lines. *f*, Cumulative latencies to first opening superimposed for sweeps with short bursts (bold trace) or long bursts. METHODS. Cell-attached recordings were obtained as previously described<sup>2</sup>. The cell membrane potential was set to zero by the bath solution's containing  $140$  mM potassium-aspartate,  $5$  mM EGTA,  $10$  mM HEPES, pH  $7.5$ . The pipette solution contained  $110$  mM  $\text{BaCl}_2$ ,  $10$  mM HEPES, pH  $7.5$ . Currents were sampled at  $5$  kHz, filtered at  $1$  kHz and analysed on a PDP11/73 lab computer. Currents were idealized by a midline crossing criterion and square-root-log histograms were constructed and fitted as described in refs 26, 27.



FIG. 3 Temporal correlation among sweeps with long or short bursts. **A**, Sequences of sweeps from three patches (*a*, *b* and *c*), each containing a single N-type channel. Sweeps were classified as nulls (no detected openings), short or long bursts as described in the legend to Fig. 2. Voltage protocol and conditions are the same as for Fig. 2. The interval between depolarizations was 5 s. **B**, Number of pairs of consecutive sweeps classified as the four possible combinations of short and long bursts in  $2 \times 2$  contingency tables. For example, the column in the lower right corner contains the pairs for which both sweeps were classified as long bursts. Pairs containing nulls were not counted. A separate table is shown for each of the experiments in **A**. The tables were used to calculate the probability that short and long bursts followed each other with the same probability as their overall proportion of the total number of sweeps in each experiment (null hypothesis). The null hypothesis was rejected for each table because  $P < 0.05$  as calculated by Fisher exact tests<sup>17</sup> or  $\chi^2$  contingency tests<sup>17</sup>. Runs analysis<sup>18,19</sup> performed on the sequences in **A** also confirmed the nonrandom occurrence of long bursts; z-values for runs of long bursts ranged from 2.7 to 17, much higher than the required minimal value of  $z = 1.64$  required to reject randomness at the  $P = 0.05$  level. To estimate average lifetimes for each gating pattern<sup>24</sup>, histograms of the number of consecutive sweeps with each gating pattern, combined from four single-channel patches, were fitted by single exponentials (not shown). The maximum likelihood estimates for the time constants and the mean durations of sojourns in each distribution (numbers in parentheses) were: nulls, 11.90 (13.2); short bursts, 5.1 (5.7) s; long bursts, 6.2 (6.2) s. The exceptionally long sequence of long bursts shown at the bottom of **A** was not included in the analysis of the duration of long bursts.



rates suggest a reversible, covalent modification of the channel protein which could explain these effects if it prevented the interaction of a cytoplasmic inactivation region with its receptor presumed to be near the inner pore of the channel protein<sup>20,21</sup>.

Spontaneous noninactivating gating has been observed in the voltage-activated  $\text{Na}^+$  channel<sup>19,22-24</sup> and A-type  $\text{K}^+$  channel<sup>25</sup> and may thus represent a general property of rapidly inactivating channels. But in contrast to native  $\text{Na}^+$  channels, in which the spontaneous loss of inactivation represents an extremely rare event (0.02% of all sweeps<sup>23</sup>), the reversible 'uncoupling' of inactivation appears to be a major functional property of N-type  $\text{Ca}^{2+}$  channels. In four patches, each containing a single N-type channel, 164 out of a total of 417 sweeps with activity (39%) showed the long, noninactivating bursting pattern. In  $\text{Na}^+$  channels, an increased proportion of nonactivating sweeps was observed after patch excision<sup>19</sup>, or in channels expressed in *Xenopus* oocytes from RNA encoding only the  $\alpha$  subunit<sup>24</sup>.

Uncoupling of microscopic inactivation also greatly increased the availability of a single N-type channel to open in response to a depolarization. Channel unavailability shows as sweeps with no openings (nulls in Fig. 3a). Within a string of sweeps with noninactivating bursts, nulls were found much less frequently than in a string of sweeps with short bursts. In four single-channel patches the probability that a short burst was followed or preceded by a null sweep (252 pairs/477 pairs = 0.53) was four times greater than the probability that a long burst was followed or preceded by a null (41/320 = 0.13). The physiological implications of the higher availability (fewer nulls) of N-type  $\text{Ca}^{2+}$  channels in their noninactivating mode are that mechanisms which promote this mode would not only decrease the rate of inactivation of whole-cell  $\text{Ca}^{2+}$  currents, but also increase the amplitude of the peak whole-cell current by increas-

ing the number of simultaneously open channels in an intact neuron or nerve terminal. The factors that can influence the equilibrium between inactivating and noninactivating modes need to be determined. Such factors would be potent modulators of  $\text{Ca}^{2+}$  currents in neuronal cells, and thus important in determining neuronal excitability and synaptic strength. □

Received 11 March; accepted 8 May 1991.

- Hess, P. A. *Rev. Neurosci.* **13**, 337-356 (1990).
- Plummer, M. R., Logothetis, D. E. & Hess, P. *Neuron* **2**, 1453-1463 (1989).
- Usovich, M. M., Porzig, H., Becker, C. & Reuter, H. *J. Physiol.* **426**, 95-116 (1990).
- Hirning, L. D. et al. *Science* **239**, 57-61 (1988).
- Lipscombe, D., Kongsamut, S. & Tsien, R. W. *Nature* **340**, 639-642 (1989).
- Swandulla, D., Carbone, E. & Lux, H. D. *Trends Neurosci.* **14**, 46-51 (1991).
- Nowicky, M. C., Fox, A. P. & Tsien, R. W. *Nature* **316**, 440-443 (1985).
- Fox, A. P., Nowicky, M. C. & Tsien, R. W. *J. Physiol.* **394**, 149-172 (1987).
- Aosaki, T. & Kasai, H. *Pflügers Arch.* **414**, 150-156 (1989).
- Jones, S. W. & Marks, T. N. *J. gen. Physiol.* **94**, 169-182 (1989).
- Kostyuk, P. G., Shuba, Y. M. & Savchenko, A. N. *Pflügers Arch.* **411**, 661-669 (1988).
- Lemos, J. R. & Nowicky, M. C. *Neuron* **2**, 1419-1426 (1989).
- Kongsamut, S., Lipscombe, D. & Tsien, R. W. *Ann. N.Y. Acad. Sci.* **560**, 312-333 (1989).
- Fisher, R. E., Gray, R. & Johnston, D. *J. Neurophysiol.* **64**, 91-104 (1990).
- Regan, L. J., Sah, D. W. Y. & Bean, B. P. *Neuron* **6**, 1-10 (1991).
- Boland, L. M. & Dingledine, R. *J. Physiol.* **420**, 223-245 (1990).
- Zar, J. H. *Biostatistical Analysis* (Prentice-Hall, Englewood Cliffs, New Jersey, 1984).
- Horn, R., Vandenberg, C. A. & Lange, K. *Biophys. J.* **45**, 323-335 (1984).
- Nilius, B. *Biophys. J.* **53**, 857-862 (1988).
- Armstrong, C. M. & Bezanilla, F. *J. gen. Physiol.* **70**, 567-590 (1977).
- Hoshi, T., Zagotta, W. N. & Aldrich, R. W. *Science* **250**, 533-538 (1990).
- Pattlak, J. B. & Ortiz, M. *J. gen. Physiol.* **86**, 89-104 (1985).
- Pattlak, J. B. & Ortiz, M. *J. gen. Physiol.* **94**, 279-301 (1989).
- Moorman, J. R., Kirsch, G. E., VanDongen, A. M. J., Joho, R. H. & Brown, A. M. *Neuron* **4**, 243-252 (1990).
- Cooper, E. & Shrier, A. *J. gen. Physiol.* **94**, 881-910 (1989).
- Sigworth, F. J. & Sine, S. M. *Biophys. J.* **52**, 1047-1054 (1987).
- McManus, O. B., Blatz, A. L. & Magleby, K. L. *Pflügers Arch.* **410**, 530-553 (1987).

ACKNOWLEDGEMENTS. This work was supported by the USPHS, and in part by the Markey Charitable Trust. P.H. is an Established Investigator of the American Heart Association.

# Spontaneous lactation is an adaptive result of pseudopregnancy

Scott R. Creel, Steven L. Monfort\*, David E. Wildt\* & Peter M. Waser

Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907, USA

\* Endocrine Research Laboratory, Conservation and Research Center, National Zoological Park, Smithsonian Institution, Front Royal, Virginia 22630, USA

LACTATION is almost exclusively associated with pregnancy and giving birth. Although lactation can be induced without a preceding pregnancy in some species, this requires exogenous hormones, artificially intense or extended suckling or both<sup>1,2</sup>. Spontaneous lactation, lactation by females that have neither been pregnant nor experimentally manipulated, is extremely unusual among eutherians<sup>3-8</sup>. Among nondomesticated animals, spontaneous lactation has been observed repeatedly only in the dwarf mongoose *Helogale parvula*<sup>9</sup>. We report here spontaneous lactation by free-living dwarf mongooses using data on urinary oestrogen conjugate concentrations ( $n = 560$ , 65 females) and body weight ( $n = 3,096$ , 25 females) from a population in Serengeti National Park, Tanzania. We use demographic data from this population to demonstrate that spontaneous lactation, and thus the endocrine phenomena that induce it, increase the evolutionary fitness of lactating females.

Dwarf mongooses live in cooperatively breeding packs (mean =  $8.9 \pm 0.4$  adults,  $n = 202$  pack-years; 1 pack-year = 1 pack monitored for 1 year) in which only a single pair reproduces, though most adults mate in synchrony<sup>9-13</sup>. Pregnancies are normally precluded in subordinate females by low oestrogen levels and low mating rates<sup>14</sup>. Subordinates occasionally (12%) become pregnant and nurse a joint litter with the dominant female, though most of the young belong to the dominant<sup>9,15</sup>. More rarely, subordinate females lactate and nurse the dominant's young, despite not having been pregnant. Including eight reported cases<sup>9</sup>, 21 spontaneous lactations have now been observed (compared with 83 subordinate pregnancies in the same period).

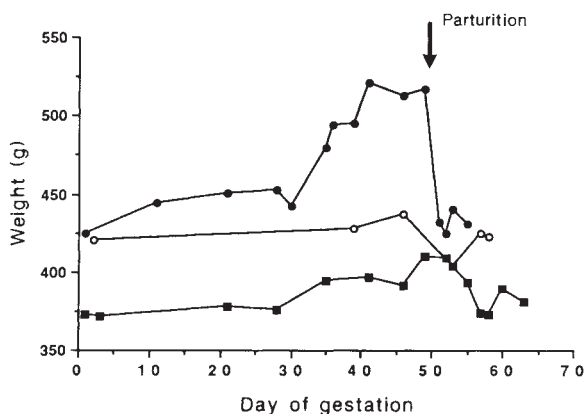


FIG. 1 Profiles of weight change for three female dwarf mongooses in a single pack, following a synchronized mating period. The 6-year-old dominant female (●) produced a litter of five on day 52. Two subordinates that subsequently lactated were not apparently pregnant (5-year-old, ○; 3-year-old, ■). Note the gradual weight loss of the 3-year-old, over a period of 6 days. Over the whole population, pregnant females exhibited no significant change in body weight between the week of oestrus and the week following parturition ( $-2.8 \pm 7.7$  g), but spontaneous lactators showed a net gain of  $36.3 \pm 7.9$  g over this interval ( $t = 2.363$ , d.f. = 19,  $P = 0.025$ ). Methods are described elsewhere<sup>23</sup>.

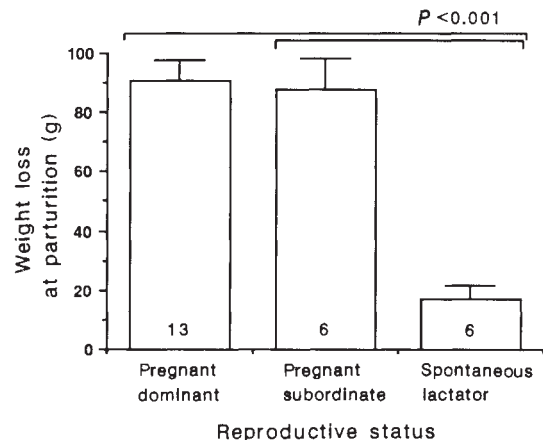


FIG. 2 Weight loss at the time of parturition. When pregnant, subordinate mongooses produced litters of the same weight as dominants ( $t = 0.247$ , d.f. = 17,  $P = 0.81$ ), but spontaneous lactators lost much less weight than either pregnant dominants ( $t = 7.256$ , d.f. = 17,  $P < 0.001$ ) or pregnant subordinates ( $t = 6.365$ , d.f. = 10,  $P < 0.001$ ). The weight loss of spontaneous lactators was only 58% of the weight loss per offspring in pregnant females. Numbers in bars indicate sample sizes. Weight change was calculated for females weighed within three days before and after the day of parturition.

Because dwarf mongooses produce litters (of 2–6 young) that weigh  $21 \pm 2\%$  of female body weight, pregnant females were easily identifiable in the field (Fig. 1), and spontaneous lactation was readily distinguishable from lactation following true pregnancy. Dominant females produced litters weighing  $90.9 \pm 6.5$  g. Litter weights of pregnant subordinates ( $88.0 \pm 10.3$  g) were statistically indistinguishable from those of dominants (Fig. 2). But spontaneous lactators lost only  $17.2 \pm 4.5$  g during the week in which they would have given birth had they been pregnant, far less than pregnant females (Figs 1 and 2). Pregnant females also lost weight mostly on a single day, whereas spontaneous lactators lost weight slowly over several days, losing as little as 1 g on the day of the parturition to which they were synchronized (Fig. 1).

These patterns of weight change suggest that spontaneous lactators may undergo pseudopregnancy<sup>16</sup>, which endocrinologically primes them for lactation. Domestic dogs occasionally lactate after pseudopregnancy<sup>8</sup>, and laboratory rats<sup>1</sup>, captive primates<sup>5</sup> and humans<sup>6</sup> may spontaneously lactate even without a preceding pseudopregnancy.

Urinary oestrogen conjugate (EC) levels support the hypothesis that spontaneous lactation follows pseudopregnancy in dwarf mongooses (Fig. 3a, b). When pregnant, mongooses' urinary EC levels increased to 70 times baseline. The EC levels of pregnant subordinates were identical to those of dominants, but the EC levels of spontaneous lactators were far lower (Fig. 3b).

Spontaneous lactators' EC profiles also differed from those of nonpregnant, nonlactating females. During the dominant female's gestation, nonlactators' EC levels remained constant, at baseline (Fig. 3a: slope =  $0.003 \pm 0.033$ , Student's  $t = 0.92$ ,  $P = 0.927$ ,  $R^2 = 0.01$ ). Spontaneous lactators, like pregnant females, showed steadily increasing EC (Fig. 3a:  $b = 0.37 \pm 0.12$ ,  $t = 3.089$ ,  $P < 0.005$ ,  $R^2 = 0.28$ ), with mean EC levels significantly higher than baseline (Fig. 3b), typical of pseudopregnancy in carnivores<sup>17-19</sup>.

Milk can be expressed from spontaneous lactators' mammae, and they can rear the orphaned young of dominants<sup>9</sup>. Both breeding females and spontaneous lactators developed visible 'udders', although those of spontaneous lactators were slightly smaller. The nipples of both became defurred synchronously. Females that lactated spontaneously first did so when they were young (mean = 2.8 years, range 2–5,  $n = 15$  females under



observation since birth), nulliparous (15/15), and still in their natal packs (17/18 lactations involving mongooses of known origin). Most spontaneous lactators were reproductively inactive during subsequent breeding bouts within that year, but 7/12 became pregnant during the next breeding season. After their first pregnancy, four females (all subordinates) exhibited a subsequent spontaneous lactation.

Both pregnant subordinates and spontaneous lactators suckled close relatives. We determined coefficients of relatedness between each subordinate and her pack's dominant male and female (the parents of most young suckled by both classes of

subordinates<sup>9,15</sup>) and analysed the angularly transformed data using factorial analysis of variance (ANOVA). Spontaneous lactators were even more closely related to dominants than were pregnant subordinates ( $F_{1,179} = 15.59$ ,  $P < 0.001$ ). Both classes of female were more closely related to the dominant female than to the dominant male ( $F_{1,179} = 33.03$ ,  $P < 0.001$ ). Subordinates tended to lactate spontaneously, rather than become pregnant, if the dominant male in their group was closely related (Fig. 4).

These patterns of relatedness may simply reflect patterns of *Helogale* demography and reproductive ontogeny. Dominants had a median tenure of 2 years, and were often succeeded by relatives<sup>10,13,20</sup>, so that the dominants in a 3-year-old's natal pack were often her close relatives. A subordinate's tendency to lactate spontaneously or to become pregnant might be conditional on her relatedness to the dominants. The one subordinate who lactated outside her natal pack helped raise young of a full sister, with whom she had dispersed<sup>9</sup>.

Because spontaneously lactating subordinates were closely related to the dominant male (Fig. 4), spontaneous lactation and pseudopregnancy might be manifestations of inbreeding depression. Both pregnant and pseudopregnant subordinates might ovulate, but embryonic mortality after inbred matings might convert pregnancies to pseudopregnancies. Because pseudopregnancy need not induce lactation<sup>8,16-19</sup>, this hypothesis does not explain why pseudopregnant females should subsequently lactate.

Whether or not pseudopregnancy is related to inbreeding, spontaneous lactation (and the pseudopregnancy that induces

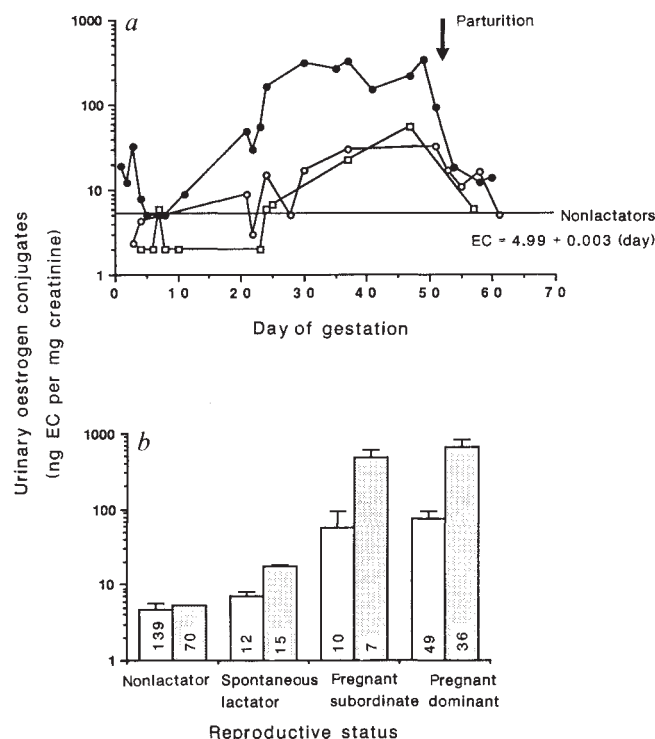


FIG. 3 *a*, Profiles of change in urinary oestrogen conjugates for three dwarf mongooses of a single pack. The line for nonlactators is a regression based on 209 points (s.e. (b)=0.03, s.e. ( $x_0$ )=0.90). During a 52-day gestation, the dominant female's EC (●) was elevated to levels typical of pregnancy in *Helogale*<sup>15</sup> (note logarithmic ordinate). Two females that mated and lactated in synchrony with the dominant (○, □) showed peak EC concentrations elevated sixfold above nonpregnant levels, but an order of magnitude below pregnant levels. In the second half of the gestation to which they were synchronized, spontaneous lactators' EC was elevated to levels similar to those of dominant females at the peak of oestrus (lactators:  $17.7 \pm 0.9$ , oestrus:  $17.9 \pm 3.5$  (ref. 14)). In the week after parturition, the EC amounts both of pregnant females and of spontaneous lactators remained elevated, and were not distinguishable from one another ( $t=1.889$ , d.f.=17,  $P=0.118$ ). These profiles are similar to those characterizing pseudopregnancy in other carnivores<sup>17-19,24</sup>, and weight data for these two females (Fig. 1) suggest that they carried no fetuses. Methods and assay validation described elsewhere<sup>14</sup>. *b*, Mean ( $\pm$ s.e.m.) urinary EC concentrations of females during dominants' gestation. Open bars show data from the first half of gestation (days 0-26); stippled bars show data from the second half (days 27-52). Pregnant dominants did not differ from pregnant subordinates in either the first ( $t=0.39$ , d.f.=57,  $P=0.70$ ) or second ( $t=0.46$ , d.f.=41,  $P=0.64$ ) halves of pregnancy. Spontaneous lactators' EC amounts were much lower than those of pregnant subordinates (overall  $t=2.80$ , d.f.=42,  $P=0.008$ ; first half  $t=1.49$ , d.f.=20,  $P=0.15$ ; second half  $t=3.36$ , d.f.=20,  $P=0.003$ ) but higher than those of nonpregnant nonlactators (overall  $t=3.50$ , d.f.=234,  $P<0.001$ ; first half, no difference; second half  $t=6.23$ , d.f.=83,  $P<0.001$ ). Like pregnant females, spontaneous lactators' EC increased significantly in the second half of the dominant's gestation (pregnant females  $t=4.45$ , d.f.=100,  $P<0.001$ ; spontaneous lactators  $t=3.25$ , d.f.=25,  $P=0.003$ ).

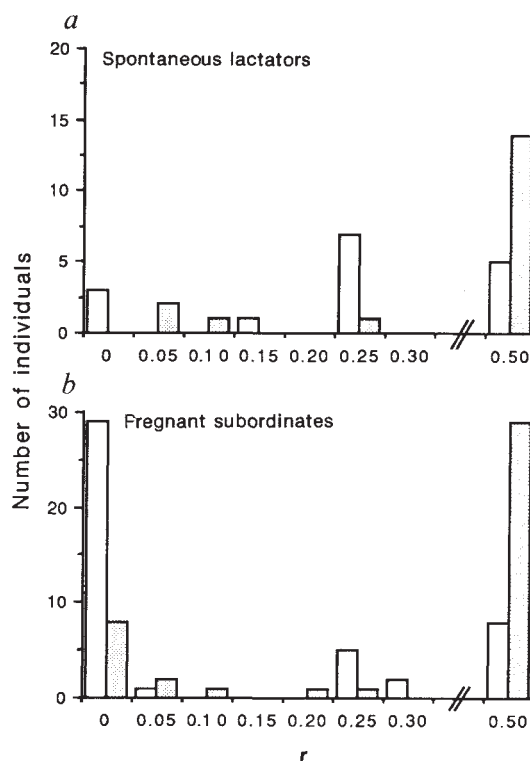


FIG. 4 *a*, Coefficients of relatedness between spontaneous lactators and dominant animals. Open bars show relatedness to the dominant male, shaded bars to the dominant female. *b*, Coefficients of relatedness between pregnant subordinates and dominant animals. Coefficients are included for all cases in which the subordinate female's parents and each of the dominants' parents are known. Pregnant subordinates were closely related to the dominant female (mean  $r \pm$  s.e. =  $0.37 \pm 0.03$ ) but less so to the dominant male ( $r=0.14 \pm 0.03$ ). In comparison, spontaneous lactators were only slightly more closely related to the dominant female ( $r=0.42 \pm 0.04$ ) but twice as closely related to the dominant male ( $r=0.28 \pm 0.05$ ).

it) could be adaptive, increasing subordinates' inclusive fitness by improving the survival of related young. Pseudopregnant subordinates suckled closely related young (relatedness coefficient,  $r=0.36$ ). After weaning, litters with spontaneous lactators were significantly larger than litters for which only the dominant female lactated ( $2.94 \pm 0.39$  versus  $1.55 \pm 0.14$  young;  $t=3.58$ ,  $P<0.001$ ).

Differences in litter size at birth were not responsible for this result: litter sizes did not differ at first count ( $3.31 \pm 0.30$  versus  $2.71 \pm 0.46$  young;  $t=1.074$ ,  $P=0.290$ ). Nor was the difference an effect of group size or of subordinate pregnancies. Each lactating nonbreeder was associated with an increase in weaned litter size of roughly one offspring, after controlling for group

size and number of pregnant subordinates (partial  $b=1.07 \pm 0.28$ ,  $t=3.85$ ,  $P<0.005$ ).

Multiplying by  $2r$ , we converted lactators' effect on relatives' fitness to offspring equivalents<sup>21,22</sup>. By nursing relatives' young, nonbreeding subordinates increased their own inclusive fitness by 0.79 offspring equivalents. Though this increment may be reduced by post-weaning mortality, it represents a substantial fitness benefit. It exceeds the benefit of becoming pregnant as a subordinate<sup>15</sup> ( $0.57 \pm 0.20$  offspring), and is 39% of the annual reproductive success of a dominant ( $2.04 \pm 0.17$ ). Pseudopregnancy, followed by spontaneous lactation, can thus substantially elevate a subordinate female's fitness. □

Received 20 February; accepted 1 May 1991.

- Jakubowski, M. & Terkel, J. *J. Reprod. Fert.* **58**, 55–60 (1980).
- Daly, M. J. *theor. Biol.* **18**, 325–345 (1979).
- Cowie, A. T. in *Hormonal Control of Reproduction* 2nd edn (eds Austin, C. R. & Short, R. V.) 195–230 (Cambridge University Press, Cambridge, 1984).
- Pereira, M. E. & Izard, M. K. *Am. J. Primatol.* **18**, 101–108 (1989).
- Holman, S. D. & Goy, R. W. *Horm. & Behav.* **14**, 348–357 (1980).
- Brown, R. E. *Pediatrics* **60**, 116–120 (1977).
- Rapaport, L. & Haight, J. J. *Mammal* **68**, 438–442 (1987).
- Smith, M. S. & McDonald, L. E. *Endocrinology* **94**, 404–412 (1974).
- Rood, J. P. *Anim. Behav.* **28**, 143–150 (1980).
- Rood, J. P. *Z. Tierpsychol.* **48**, 277–287 (1978).
- Rood, J. P. in *Advances in the Study of Mammalian Behavior* (eds Eisenberg, J. F. & Kleiman, D. G.) 454–458 (American Society of Mammalogists, Lawrence, Kansas, 1983).
- Rasa, O. A. E. *Z. Tierpsychol.* **43**, 337–406 (1977).
- Rasa, O. A. E. *Adv. Study Behav.* **17**, 121–163 (1987).
- Creel, S. R., Creel, N. M., Wildt, D. E. & Monfort, S. L. *Anim. Behav.* (in the press).

- Creel, S. R. & Waser, P. M. *Behav. Ecol.* (in the press).
- Nalbandov, A. V. *Reproductive Physiology of Birds and Mammals* (Freeman, San Francisco, 1976).
- Concannon, P. W., Powers, M. E., Holder, W. & Hansel, W. *Biol. Reprod.* **16**, 517–526 (1977).
- Verhage, H. G., Beamer, N. B. & Brenner, R. M. *Biol. Reprod.* **14**, 579–585 (1976).
- Seal, U. S., Plotka, E. D., Packard, J. M. & Mech, L. D. *Biol. Reprod.* **21**, 1057–1066 (1979).
- Rood, J. P. *Anim. Behav.* **39**, 566–572 (1990).
- West-Eberhard, M. J. *Q. Rev. Biol.* **50**, 1–33 (1975).
- Brown, J. L. & Brown, E. R. in *Natural Selection and Social Behavior: Recent Research and New Theory* (eds Alexander, R. D. & Tinkle, D. W.) 244–256 (Chiron Press, New York, 1981).
- Creel, S. R. & Creel, N. M. *Behav. Ecol. Sociobiol.* (in the press).
- Hadley, J. C. *J. Reprod. Fert.* **44**, 453–460 (1975).

ACKNOWLEDGEMENTS. We thank N. Creel for her help, J. H. and J. Rood for demographic data, K. Hirji (SWRI), D. Babu (TANAPA), B. Maragesi (SNP) and H. Nkya (SWRC) for permission to work in Serengeti, M. East, H. Hofer and other SWRC friends for help in the field, P. Amarasekare, L. Elliott, R. Howard, B. Keane, D. Minchella, W. Piper and P. Rabenold for helpful comments. This work was supported by the National Geographic Society and the National Science Foundation.

## The B-cell surface protein CD72/Lyb-2 is the ligand for CD5

Hilde Van de Velde\*, Ilka von Hoegen†, Wei Luo\*, Jane R. Parnes† & Kris Thielemans\*‡

\* Division of Haematology–Immunology, Medical School of the Vrije Universiteit Brussel, B-1090 Brussels, Belgium

† Division of Immunology and Rheumatology,

Stanford University Medical Center, Stanford, California 94305, USA

THE glycoprotein CD5 is expressed on the surface membrane of all mature T cells<sup>1</sup> and a small proportion of B lymphocytes<sup>1,2</sup>. Its exact role in immune interactions is still unknown. Studies<sup>3–10</sup> indicate that CD5 functions both in mice and humans as a receptor, delivering co-stimulatory signals to T cells in a manner similar to CD2 (ref. 11) and CD28 (ref. 12). Anti-CD5 antibodies stimulate both T-cell proliferation mediated by CD3 in association with the T-cell receptor and secretion of interleukin-2 and expression of its receptor, as well as inducing an increase in intracellular  $Ca^{2+}$  concentration (refs 5–10). To identify the ligand for CD5 we purified the human CD5 protein, labelled it with biotin and used it as a probe. Here we report that CD5 specifically interacts with the cell-surface protein CD72 exclusive to B cells. This interaction is blocked by anti-CD72 antibodies, but not by any other anti-B-cell antibodies. Moreover, non-B cells (mouse L-cell fibroblasts and human Jurkat T cells) expressing a transfected human CD72 complementary DNA could bind to the CD5–biotin conjugate. The results demonstrate that the B-cell surface protein CD72 (Lyb-2 in mice) is the ligand for CD5.

The communication between T cells and B cells is mediated by receptors, either directly by cell–cell contact or by soluble factors (cytokines or lymphokines). T lymphocytes can be activated through several pathways, one of which is stimulated by monoclonal antibodies to CD5 (refs 5–10). On the basis of procedures to purify leukocyte antigens<sup>13,14</sup>, we purified human

CD5 membrane protein from a cell lysate of a human T-ALL cell line (CEM) expressing CD5 (Fig. 1). By using two different antibodies, CRIS-1 (IgG2a) and OKT1 (IgG1), recognizing distinct epitopes of the CD5 protein, we could detect CD5 in dilute solutions in an enzyme-linked immunosorbent assay (ELISA) (Fig. 1a). The purified CD5 migrated to a position corresponding to its expected relative molecular mass of 67,000 ( $M_r$  67K) on SDS-PAGE (Fig. 1b). The purified protein was biotinylated as described<sup>15</sup>. The CD5–biotin conjugate reacted exclusively with seven anti-CD5 antibodies (Fig. 1c, Leu-1 OKT-1 CRIS-1; not shown, B11a, RFT-1, MEM-32 and B11b)<sup>16</sup>, but not with any of 10 class-matched control antibodies examined (for example, OKT-3).

Although CD5 could be a receptor for a soluble factor<sup>5</sup>, it now seems more likely that it interacts directly with a counter-receptor on the surface of accessory cells<sup>10</sup>. Therefore, we searched for a ligand for CD5 by first assessing CD5 binding to cell-surface structures using biotin-labelled CD5 and the streptavidin system. Different haematopoietic cell lineages were examined (Fig. 2). Only pre-B and B-cell lines (Fig. 2, NALM-6, RAJ1; not shown, JOK, DAUDI, HPI and SU-DHL) showed binding to the CD5–biotin conjugate. The conjugate did not react with T cells (Fig. 2, CEM; not shown, MOLT-4 and Jurkat), monocytoid cells (Fig. 2, HL60; not shown, U937 and K562) or freshly isolated granulocytes (data not shown). Plasma cell lines (Fig. 2, U266; not shown, LP-1 and RPMI 8266) were also unreactive, indicating that the ligand for CD5 disappears from the membrane during the final differentiation of B lymphocytes. Freshly isolated CD19<sup>+</sup> peripheral blood B cells (data not shown) and CD19<sup>+</sup> tonsil B cells (Fig. 2) also bound CD5–biotin, but lost this reactivity after stimulation *in vitro* with phorbol esters and/or T cell-conditioned medium (data not shown). The binding of B lymphocytes to CD5–biotin was inhibited by co-incubation with an excess of anti-CD5 antibody (Leu-1) but not with a class-matched control antibody (data not shown).

To identify the molecule on pre-B and B cells that interacts with CD5, we investigated which of a panel of 129 anti-B-cell antibodies (coded B1 to B129 from the Fourth Workshop on Leucocyte Differentiation Antigens<sup>17</sup>) could block this inter-

‡ To whom correspondence should be addressed.



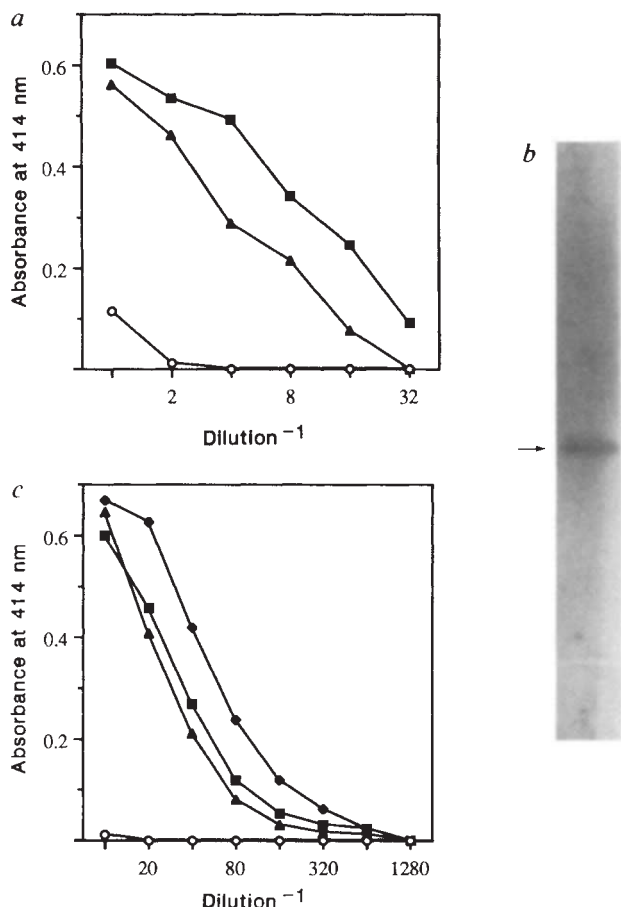


FIG. 1 Purification of CD5. *a*, A sandwich ELISA was used to specifically detect the presence of CD5 in a cell lysate of human CD5<sup>+</sup> CEM cells (▲), in the protein solution eluted from the anti-CD5 affinity matrix (■) or in a cell lysate of a CD5<sup>-</sup> human B cell line, SU-DHL (○). *b*, SDS-PAGE shows that the 67K CD5 protein was purified to homogeneity. *c*, Biotinylated CD5 reacts specifically in an ELISA with anti-CD5 antibodies CRIS-1 (■), Leu-1 (◆) or CRIS-1 (▲), but not with control antibody OKT-3 (○).

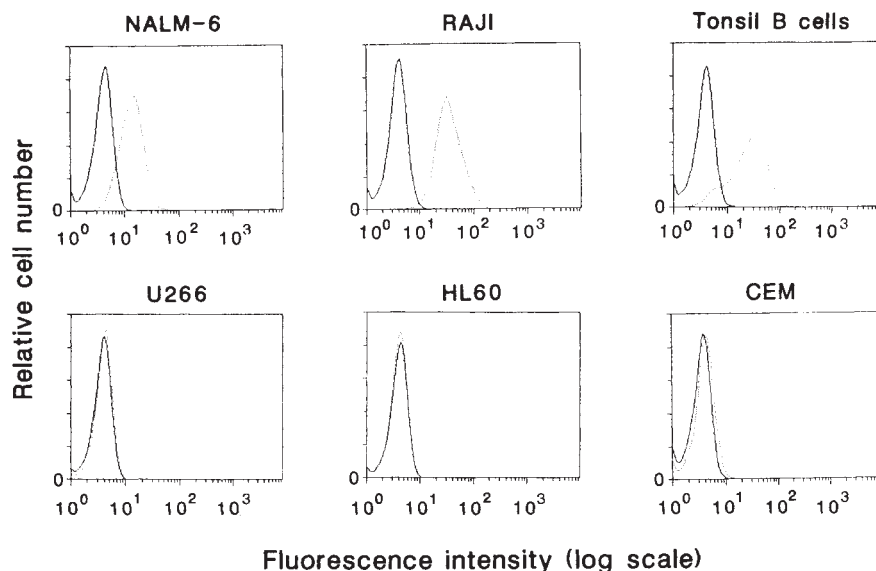
**METHODS.** Human CD5<sup>+</sup> CEM cells cultured *in vitro* were lysed by resuspension of the cell pellet in ice-cold TSA buffer ( $5 \times 10^7$  cells ml<sup>-1</sup>; TSA buffer is composed of 25 mM Tris-HCl, pH 7.4, 10 mM NaCl, 0.025 % NaN<sub>3</sub>, 5 mM MgCl<sub>2</sub>, 1 mM PMSF, 1 mM pepstatin A, 10 mM iodoacetamide, 0.2 U ml<sup>-1</sup> aprotinin and 1% Triton X-100). The lysed cell suspension was first centrifuged at 10,000g for 30 min and then at 100,000g for 30 min at 4 °C. The resulting supernatant was then filtered through a 0.2-μm nitrocellulose filter and applied to three columns: first, Sepharose-4B beads; second, Sepharose-4B to which polyclonal mouse immunoglobulin was bound, and third, Sepharose-4B coupled with anti-human antibody (Leu-1). The affinity column was washed extensively and bound protein was eluted with 3.5 M NaSCN containing 0.05% Triton X-100. Fractions containing protein were pooled and dialysed against 1,000 times the volume of PBS medium. The protein content was determined using the Bio-Rad Protein Assay system. Usually,  $5 \times 10^9$  cells yielded about 150 μg CD5. For the sandwich ELISA, microtitre plates were coated with anti-CD5 antibody CRIS-1 (lgG2a; 10 μg ml<sup>-1</sup> in PBS, 50 μl per well). After washing the plates, serial dilutions of a cell lysate or eluted protein were incubated for 1 h at 4 °C. After removal of unbound proteins, CD5 was detected with a second anti-CD5 antibody (OKT-1; IgG1) and peroxidase-conjugated goat anti-mouse IgG1 antibodies (Southern Biotechnology, Alabama). Eluted protein was analysed by 10% SDS-PAGE and silver staining according to the Bio-Rad instructions. Purified CD5 was biotinylated as described<sup>15</sup>, and the specific reactivity with anti-CD5 antibodies was determined. ELISA plates were coated with anti-CD5 or control antibodies. Serial dilutions of CD5-biotin were then added and the reactivity was detected with streptavidin-peroxidase conjugate. Wells coated with control antibodies directed at cell-surface antigens other than CD5 were consistently negative.

action. Only four antibodies inhibited the binding of B cells to CD5-biotin (Fig. 3; code numbers B6: S-HLC2; B24: J3-109; B64: BU40; B81: BU41). They were the only ones in the panel that were directed against CD72. Further proof for the specific CD5-CD72 interaction was obtained by staining of non-B cells transfected with human CD72 cDNA<sup>18,19</sup>. Transfection of the human CD72 clone into mouse L-cell fibroblasts results in cell-surface expression of human CD72 and binding of CD5-biotin (Fig. 4). By contrast, untransfected control cells did not react with either anti-CD72 antibody or CD5-biotin. Similar findings were obtained using Jurkat T cells transfected with

human CD72 cDNA (data not shown). Binding of CD5-biotin to the CD72 transfectants was inhibited by unlabelled anti-CD72 or anti-CD5 antibodies (data not shown). We have obtained similar results for binding of purified mouse CD5 to the mouse homologue of CD72, Lyb-2 (unpublished results).

These data demonstrate an interaction between CD5 and CD72. Human CD72 is a glycoprotein of  $M_r$  42-43K and is expressed as a disulphide-linked homodimer on cells of the B-lymphocyte lineage<sup>17-20</sup>. It is present on pre-B and mature B cells, but is absent once these cells differentiate into plasma cells<sup>17-21</sup>. Human CD72 is the homologue of the mouse B-cell

FIG. 2 Flow cytometry of different leukocyte lineages with CD5-biotin. The human cell lines NALM-6 (pre-B cell line), RAJI (B-cell line), U266 (plasma cell line), HL60 (monocytoid cell line) and CEM (T-cell line) and tonsil B lymphocytes were each incubated for 30 min at 4 °C with 10 μg per 10<sup>6</sup> cells BSA-biotin (solid line) or CD5-biotin (dotted line). After washing with PBS-3% BSA, the cells were incubated with streptavidin-phycoerythrin conjugate (1/50; Becton Dickinson, Belgium), washed again and analysed using a FAC-Star flowcytometer.



differentiation antigen Lyb-2 (ref. 19). Complementary DNAs encoding both mouse and human CD72/Lyb-2 have been cloned and sequenced; the encoded proteins are type II integral membrane proteins whose external domain is homologous to that of asialoglycoprotein receptors and CD23, the low-affinity Fc receptor for IgE (refs 18, 22). Antibodies specific for human and mouse CD72/Lyb-2 can induce B-cell proliferation either alone or in combination with B-cell growth factors<sup>23-27</sup>, and can induce class II expression<sup>24,28</sup> and small increases in intracellular  $Ca^{2+}$  concentration<sup>23,28</sup> in human and mouse B cells, respectively. Further, antibodies specific for mouse Lyb-2 can also block the differentiation of B cells into antibody-secreting cells in response to antigens dependent on T cells<sup>29,30</sup>. These data suggest that CD72/Lyb-2 is a receptor for a B-cell growth factor. But there is no evidence as to whether a soluble or cell-surface ligand binds to CD72/Lyb-2.

Further exploration of the CD5-CD72 interaction is likely to give insight into how T and B lymphocytes communicate directly by cell-cell contact and how this interaction contributes to T- and B-cell activation and proliferation. Moreover, an understanding of this interaction might provide clues to the proposed immunoregulatory role of the CD5<sup>+</sup> B-cell lineage. It has been postulated that these cells help in the activation and development of conventional CD5<sup>-</sup> B cells<sup>32</sup>, processes in which CD5-CD72 interaction could be involved. As B-cell lymphocytic leukaemia cells express both CD5 and its ligand/receptor CD72 (ref. 17 and our unpublished observations), the CD5-CD72 interaction

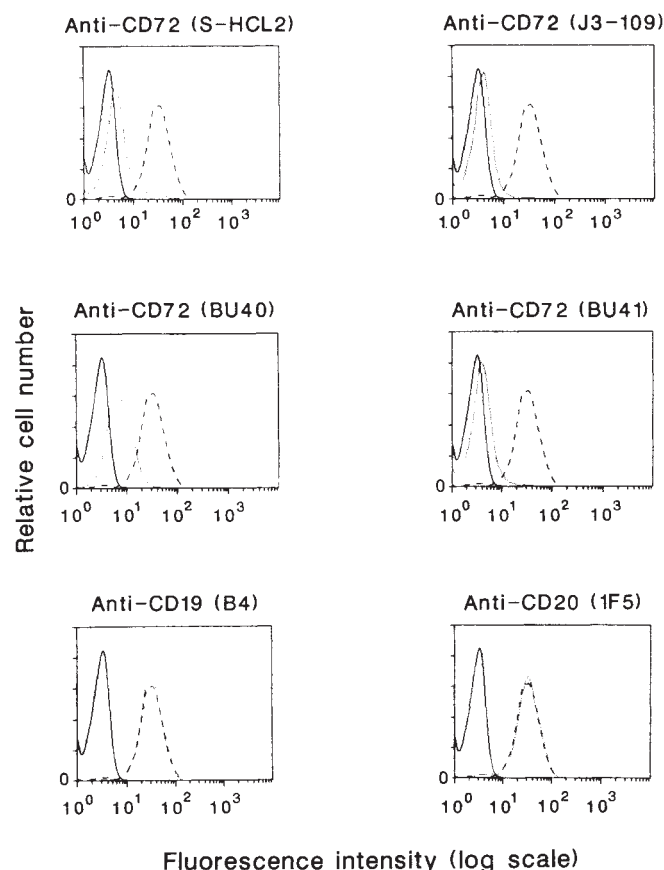


FIG. 3 Inhibition of CD5-biotin staining by anti-CD72 antibodies. RAJI B cells were incubated with BSA-biotin (solid line), CD5-biotin (broken line) or with either unlabelled anti-CD72 or control antibodies (specificity and clone designation are indicated above the histograms) followed by CD5-biotin (dotted line) for 30 min at 4 °C. The cells were washed and incubated for 30 min at 4 °C with streptavidin-phycoerythrin conjugate. After a final wash the cells were analysed.

#### Untransfected L cells

#### Human CD72 cDNA transfected L cells

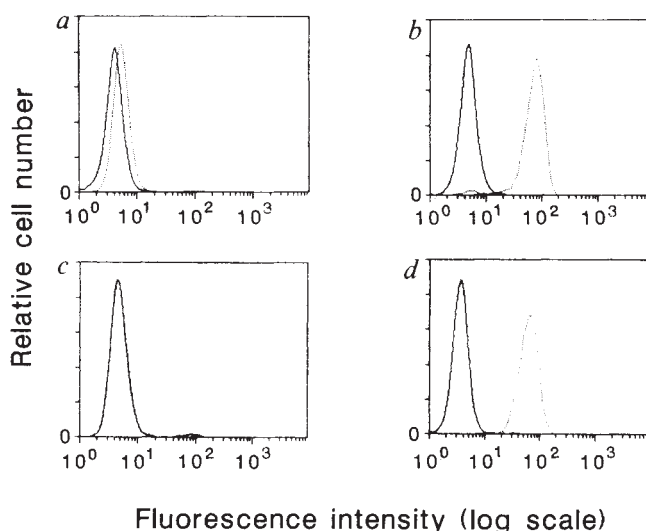


FIG. 4 Flow cytometry of CD72 cDNA-transfected cells with CD5-biotin. A cDNA clone encoding human CD72 was subcloned into the expression vector pHAPr-neo-1 (ref. 31) and transfected into mouse L cells as described<sup>18</sup>. Untransfected (a and c) and transfected (b and d) L cells were incubated at 4 °C for 30 min with BSA-biotin (solid line, a and b), CD5-biotin (dotted line a and b), control IgG1 (solid line, c and d) or anti-CD72 antibody (clone A11, IgG1<sup>19</sup>, dotted line, c and d). After washing the cells with PBS-3% BSA, bound reagents were revealed by a second incubation with a conjugate of streptavidin-phycoerythrin (a and b) or fluorescein-goat anti-mouse antibodies (c and d).

might contribute to autostimulatory growth of these transformed cells<sup>33</sup>.

Received 2 April; accepted 29 April 1991.

- Wang, C. Y., Good, R. A., Ammizate, P., Dymberg, G. & Evans, R. *J. exp. Med.* **151**, 1539-1544 (1980).
- Hayakawa, K. & Hardy, R. R. *A. Rev. Immun.* **6**, 197-218 (1988).
- Jones, N. H. *et al. Nature* **323**, 346-349 (1986).
- Huang, H. S., Jones, N. H., Strominger, J. L. & Herzenberg, L. A. *Proc. natn. Acad. Sci. U.S.A.* **84**, 204-208 (1987).
- Ceuppens, J. L. & Baroja, M. L. *J. Immun.* **137**, 1816-1821 (1986).
- Ledbetter, J. A., June, C. H., Grosmaire, L. S. & Rabinovitch, P. S. *Proc. natn. Acad. Sci. U.S.A.* **84**, 1384-1388 (1987).
- Nishimura, Y. *et al. Eur. J. Immun.* **18**, 747-753 (1988).
- Vervilghen, J., Vandesande, R., Vandenberghe, P. & Ceuppens, J. L. *Cell. Immun.* **131**, 109-119 (1990).
- Spertini, F., Stohli, W., Ramesh, N., Moody, C. & Geha, R. S. *J. Immun.* **146**, 47-52 (1991).
- Vandenberghe, P. & Ceuppens, J. L. *Eur. J. Immun.* **21**, 251-259 (1991).
- Springer, T. A. *Nature* **346**, 425-434 (1990).
- Linsley, P. S., Clark, E. A. & Ledbetter, J. A. *Proc. natn. Acad. Sci. U.S.A.* **87**, 5031-5035 (1990).
- Kürzinger, K. & Springer, T. A. *J. biol. Chem.* **257**, 12412-12418 (1982).
- Marlin, S. D. & Springer, T. A. *Cell* **51**, 813-819 (1987).
- Taki, S., Shimamura, T., Abe, M., Shirai, T. & Takahara, Y. *J. Immunol. Meth.* **122**, 33-41 (1989).
- McMichael, A. J. & Gotch, F. M. in *Leucocyte Typing III* (ed. McMichael, A. J.) 31-62 (Oxford University Press, Oxford, 1987).
- Dörken, B., Möller, P., Pezzutto, A., Schwartz-Albiez, R. & Moldenhauer, G. in *Leucocyte Typing IV* (eds Knapp, W. *et al.*) 15-32 (Oxford University Press, Oxford, 1989).
- Von Hoegen, I., Nakayama, E. & Parnes, J. R. *J. Immun.* **144**, 4870-4877 (1990).
- Von Hoegen, I., Hsieh, C., Schwartz, R., Francke, U. & Parnes, J. R. *Eur. J. Immun.* (in the press).
- Schwartz, R. & Stein, H. in *Leucocyte Typing IV* (eds Knapp, W. *et al.*) 100-102 (Oxford University Press, Oxford, 1989).
- Dörken, B., Möller, P., Pezzutto, A., Schwartz-Albiez, R. & Moldenhauer, G. in *Leucocyte Typing IV* (eds Knapp, W. *et al.*) 99-100 (Oxford University Press, Oxford, 1989).
- Nakayama, E., von Hoegen, I. & Parnes, J. R. *Proc. natn. Acad. Sci. U.S.A.* **86**, 1352-1356 (1989).
- Pezzutto, A., Dörken, B., Valentine, M. A., Shu, G. L. & Clark, E. A. in *Leucocyte Typing IV* (eds Knapp, W. *et al.*) 178-182 (Oxford University Press, Oxford, 1989).
- Kamal, M., Katira, A. & Gordon, J. *Eur. J. Immun.* (in the press).
- Subbarao, B. & Mosier, D. E. *J. Immun.* **140**, 2033-2037 (1983).
- Subbarao, B. & Melchers, F. *Curr. Topics Microbiol. Immun.* **113**, 72-76 (1984).
- Snow, E. C., Mond, J. J. & Subbarao, B. *J. Immun.* **137**, 1793-1796 (1986).
- Subbarao, B., Morris, J. & Baluyut, A. R. *Cell. Immun.* **112**, 329-342 (1988).
- Yakura, H., Shen, F.-W., Kaemer, M. & Boyse, E. A. *J. exp. med.* **153**, 129-135 (1981).
- Subbarao, B. & Mosier, D. E. *Immunol. Rev.* **69**, 81-97 (1982).
- Gunning, P., Leavitt, J., Muscat, G., Ng, S.-Y. & Kedes, L. *Proc. natn. Acad. Sci. U.S.A.* **84**, 4831-4837 (1987).



32. Hardin, J. A. *et al.* *Cell Immun.* **126**, 304–321 (1990).  
 33. Kawamura, N. *et al.* *J. clin. Invest.* **78**, 1331–1338 (1986).

ACKNOWLEDGEMENTS. This work was supported by the Fund for Medical Research (Belgium) (K.T.) and the NIH (J.R.P.). H.V.d.V. is a fellow and K.T. a Research Associate of the NFWO. I.v.H. is a postdoctoral fellow of the Arthritis Foundation. J.R.P. is an Established Investigator of the American Heart Association.

## Uniparental paternal disomy in a genetic cancer-predisposing syndrome

I. Henry\*, C. Bonaiti-Pellié†, V. Chehensse\*,  
 C. Beldjord‡, C. Schwartz§, G. Utermann¶ & C. Junien\*

\* INSERM U73, Génétique et Pathologie Foetale, Château de Longchamp, 75016 Paris, France

† INSERM U155, Epidémiologie Génétique, Château de Longchamp, 75016 Paris, France

‡ INSERM U129, Génétique et Pathologie Moléculaire, Cochin Port-Royal, 75014 Paris, France

§ Greenwood Genetic Center, Greenwood, South Carolina 29646, USA

¶ University of Innsbruck, A-6020, Innsbruck, Austria

**THE 11p15.5 region of human chromosome 11 seems to contain a locus or loci involved in congenital overgrowth anomalies as well as in the genesis of many tumours associated with the Beckwith-Wiedemann syndrome (BWS)<sup>1–6</sup>. Given the unusual differential parental allele involvement in the different aetiological forms of BWS<sup>5,7</sup> and the loss of maternal alleles in associated tumours<sup>8–10</sup>, we have now used 11p15.5 markers to determine the parental origin of chromosome 11 in eight sporadic cases of BWS. Probands in three informative families had uniparental paternal disomy for**

**region 11p15.5. Further, an overall greatly increased frequency of homozygosity for several 11p15.5 markers in 21 sporadic BWS patients suggests that isodisomy probably accounts for an even higher proportion of BWS sporadic cases. This demonstrates that uniparental paternal disomy can be associated with a genetic cancer-predisposing syndrome.**

All 21 sporadic BWS individuals studied had a typical phenotype of BWS, that is exomphalos, macroglossia, gigantism, hypoglycaemia and visceromegaly. Four had developed a Wilms' tumour (patients BW9P, BW11P, BW18P and BW21P). The constitutional karyotype analysis failed to reveal a chromosomal aberration. Restriction fragment length polymorphism (RFLP) analysis was done using nine RFLPs at six loci ranging from 11pter to 11p15.5 in the following order: HRAS1-(IGF2-INS-TH)-D11S12-HBB-HBBP. The genotypes of eight BWS patients and of 14 matched parents were determined (Table 1). In three BWS patients (BW11P, BW15P and BW21P), we have shown that there was an absence of maternal contribution for at least one marker mapping to 11p15.5 (Fig. 1a). Gene copy number determination revealed that all three patients had inherited two paternal copies (Fig. 1b, c, d) and thus displayed paternal disomy. Isodisomy rather than heterodisomy can be demonstrated only for BW21P (Fig. 1a). The region for which the patients are disomic includes INS-IGF2 and extends to HRAS1 (BW11P) and to HBBP (BW21P).

To test whether being uniparental for 11p15 disomy could be a common, yet unrecorded genetic event, we compared the genotypes of 18 unrelated control individuals with the genotypes of their parents. We did not detect uniparental disomy in this control population (data not shown). Therefore the occurrence of uniparental disomy in three out of eight sporadic BWS patients is probably not fortuitous (3/8 versus 0/18; *P*, 0.02, one-tailed test). A few cases with a duplication of 11p15.5 sequences have been reported<sup>1,2</sup>. All were almost exclusively of

TABLE 1 Genotypes of eight BWS patients and their parents for nine 11p15.5 RFLPs

|       | HRAS1<br><i>Bam</i> HI | TH<br><i>Taq</i> I | INS<br><i>Rsa</i> I | INS<br><i>Hind</i> III | IGF2<br><i>Sac</i> I | IGF2<br><i>Av</i> all | D11S12<br><i>Msp</i> I | HBB<br><i>Av</i> all | HBBP<br><i>Hinc</i> II |
|-------|------------------------|--------------------|---------------------|------------------------|----------------------|-----------------------|------------------------|----------------------|------------------------|
| BW11P | 1/1                    | 2/2                | 1/1                 | 1/1                    | 2/2                  | 1/1                   | 2/2                    | 2/2                  | 1/1                    |
| BW11M | 2/2                    | 2/2                | 1/2                 | 1/2                    | 1/1                  | 1/1                   | 1/2                    | 2/2                  | 1/2                    |
| BW11F | 1/1                    | 2/2                | 1/1                 | 1/1                    | 2/2                  | 1/1                   | 1/2                    | 2/2                  | 1/2                    |
| BW12P | 2/2                    | 2/2                | 1/1                 | 1/2                    | 1/1                  | 1/1                   | 1/2                    | 1/1                  | 2/2                    |
| BW12M | 1/2                    | 2/2                | 1/1                 | 1/2                    | 1/1                  | 1/2                   | 1/1                    | 1/2                  | 2/3                    |
| BW13P | 1/2                    | 2/2                | 2/2                 | 1/2                    | 2/2                  | 1/1                   | 1/2                    | 2/2                  | 1/2                    |
| BW13M | 1/2                    | 2/2                | 2/2                 | 1/2                    | 2/2                  | 1/1                   | 1/2                    | 2/2                  | 2/3                    |
| BW13F | 1/2                    | 2/2                | 2/2                 | 2/2                    | 2/2                  | 1/1                   | 1/2                    | 1/2                  | 1/1                    |
| BW14P | 1/2                    | 2/2                | 1/1                 | 2/2                    | 1/2                  | 1/1                   | 1/2                    | 2/2                  | 1/1                    |
| BW14M | 2/2                    | 2/2                | 1/2                 | 2/2                    | 1/2                  | 1/2                   | 1/2                    | 2/2                  | 1/3                    |
| BW14F | 1/2                    | 2/2                | 1/2                 | 1/2                    | 2/2                  | 1/1                   | 1/2                    | 2/2                  | 1/1                    |
| BW15P | 2/2                    | 1/2                | 2/2                 | 2/2                    | 2/2                  | 1/1                   | 1/1                    | 2/2                  | 1/1                    |
| BW15M | 2/2                    | 2/2                | 1/1                 | 1/2                    | 2/2                  | 1/1                   | 1/2                    | 1/2                  | 1/1                    |
| BW15F | 2/2                    | 1/2                | 2/2                 | 1/2                    | 2/2                  | 1/1                   | 1/2                    | 2/2                  | 1/3                    |
| BW16P | 1/2                    | 2/2                | 2/2                 | 1/2                    | 1/2                  | 1/2                   | 2/2                    | 1/2                  | 1/3                    |
| BW16M | 2/2                    | 2/2                | 2/2                 | 1/2                    | 2/2                  | 1/1                   | 1/2                    | 2/2                  | 1/3                    |
| BW17P | 2/2                    | 2/2                | 1/2                 | 1/2                    | 2/2                  | 1/2                   | 2/2                    | 2/2                  | 1/3                    |
| BW17M | 1/2                    | 2/2                | 1/2                 | 1/2                    | 1/2                  | 1/2                   | 1/2                    | 2/2                  | 1/2                    |
| BW17F | 2/2                    | 2/2                | 1/1                 | 1/2                    | 1/2                  | 1/1                   | 2/2                    | 2/2                  | 1/3                    |
| BW21P | 2/2                    | 2/2                | 1/1                 | 1/1                    | 2/2                  | 1/1                   | 2/2                    | 2/2                  | 2/2                    |
| BW21M | 1/2                    | 2/2                | 2/2                 | 2/2                    | 1/2                  | 1/2                   | 1/2                    | 1/2                  | 1/3                    |
| BW21F | 2/2                    | 2/2                | 1/1                 | 1/2                    | 2/2                  | 1/1                   | 2/2                    | 1/2                  | 1/2                    |

DNA was isolated from blood and/or lymphoblastoid cell lines. Southern blot analysis was performed as previously described<sup>16</sup>. Probes and restriction enzymes used for RFLP analysis were: HRAS1 (c-Ha-ras) with *Bam*HI, TH (Tyrosine Hydroxylase) with *Taq*I, INS (Insulin) with *Rsa*I and *Hind*III, IGF2 (Insulin-like Growth Factor II) with *Sac*I and *Av*all, D11S12 (pADJ762) with *Msp*I, HBB ( $\beta$ -globin) with *Av*all, and HBBP ( $\beta$ -globin, pseudogene) with *Hinc*II<sup>17</sup>. These markers were chosen because they were duplicated in two BWS patients with cytological duplications of 11p15.5 (ref. 3), and revealed loss of heterozygosity in several tumours<sup>18–25</sup>. RFLP alleles were named 1, 2 or 3 according to decreasing length. Parents were as follows: BW11M and BW11F are the mother and the father of patient BW11P. Genotypes in boxes: Loci for which the patients failed to inherit maternal alleles.

paternal origin. Further, in several families with the autosomal dominant syndrome, genetic linkage of the BWS locus to markers within 11p15.5 has been established<sup>4,5</sup>. In these families there is an excess of female carriers, suggesting a sex-dependent mode of transmission<sup>5,7</sup> partly due to a higher penetrance in individuals born to female carriers (C. Moutou, personal communication).

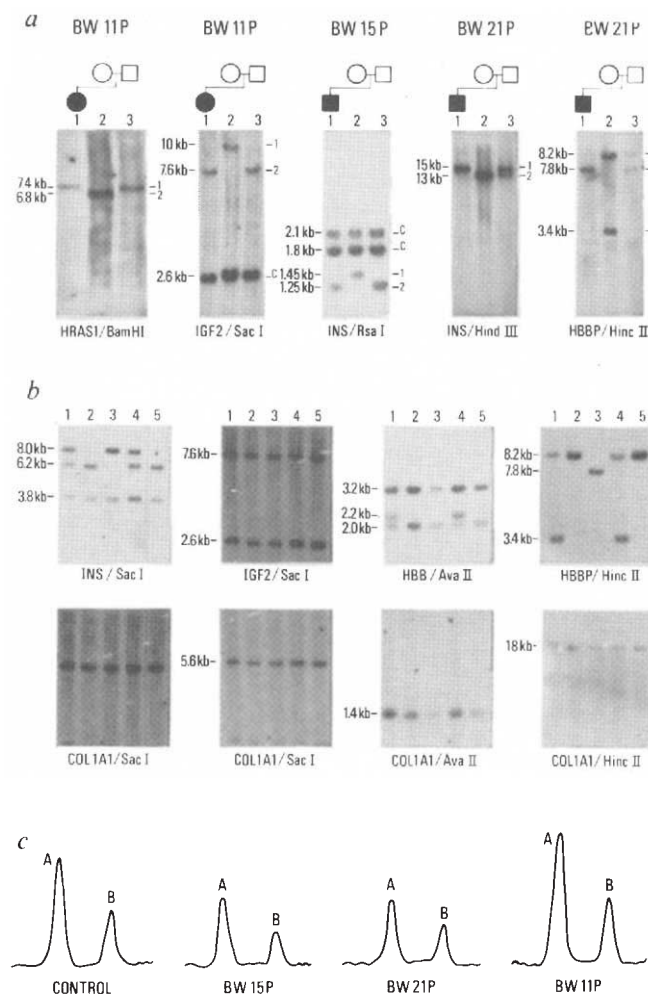
To determine whether isodisomy is a common event in sporadic cases of BWS, we compared the distribution of the different genotypes in the 21 unrelated BWS patients with that in 36 unrelated controls. There was a significant distortion of the genotype distribution in BWS individuals (Table 2). BWS patients were more frequently homozygous for four contiguous markers taken together, *INS/RsaI*, *INS/HindIII*, *IGF2/SacI* and *IGF2/AvaII*, than controls. The distortion suggests that uniparental isodisomy probably accounts for an even higher proportion of sporadic cases of BWS. Given the informativeness and the distance between them, the *INS/IGF2* region can be considered as part of the region of disomy most probably common to the different patients.

The event triggering malignant proliferation in the same type of tumours as those observed in 10% of BWS patients, is a loss of 11p15.5 alleles. The alleles lost are always of maternal

origin<sup>8-10</sup>. Of four BWS patients who developed a tumour, two, BW11P and BW21P, displayed paternal disomy. Paternal disomy with absence of maternal alleles is thus compatible with the development of Wilms' tumour. As patient BW15P, who is also disomic and has not developed a tumour at age five, this may suggest that the absence of maternal 11p15.5 alleles observed in many tumours is probably not sufficient for tumour development. One must await the cloning of the gene(s) to determine whether the remaining paternal allele(s) is functional or altered and the nature of the differential imprinting.

We have shown that BWS can occur following erroneous events during meiosis or after fertilization leading to complete or partial uniparental isodisomy (or heterodisomy), even in the absence of obvious clinical signs in the parents. There are two possible explanations to account for the occurrence of BWS in children with paternal disomy. First, BWS in the three disomic cases could be due to inheritance of a recessive paternal mutation as reported in two patients with cystic fibrosis (CF) and short stature<sup>11,12</sup>. Second, the BWS locus (or loci) more probably undergoes differential genomic imprinting<sup>13</sup>. Although the number of cases reported here is insufficient to demonstrate that uniparental disomy is always of paternal origin in BWS, our findings are similar to data in two phenotypically different

FIG. 1 Paternal uniparental disomy in three BWS patients with cytogenetically normal chromosome 11. *a*, Absence of maternal alleles for 3 BWS patients: Southern blot experiments. BW11P, Paternal origin of *HRAS1/BamHI* and *IGF2/SacI* in patient BW11P. The 7.4-kilobase (kb) *HRAS1* allele and the 7.6 kb *IGF2* allele is shared by the patient BW11P (lane 1) and her father BW11F (lane 3), whereas the patient inherited neither the 6.8-kb *HRAS1* allele nor the 10-kb *IGF2* allele from her mother BW11M (lane 2). The 2.6-kb *IGF2* band is a constant band. BW15P, Paternal origin of *INS/RsaI* in patient BW15P. The same 1.45-kb allele is shared by the patient BW15P (lane 1) and his father BW15F (lane 3), whereas the patient (BW15P) did not inherit a 1.25-kb allele from his mother BW15M (lane 2). The 2.1 and 1.8 kb bands are constant bands. BW21P, Paternal origin of *INS/HindIII* and *HBBP/HincII* in patient BW21P. The patient BW21P (lane 1) only displays the 15-kb *INS* alleles from his heterozygous father (lane 3, 15-kb and 13-kb alleles). This patient did not inherit a 12-kb *INS* allele from his mother BW21M, homozygous for this allele (lane 2). Similarly the patient displays only one 7.8-kb *HBBP* allele from his heterozygous father and did not inherit the 8.2-kb or 3.4-kb allele from his mother. Isodisomy rather than heterodisomy can account for this pattern. Using non-11p highly polymorphic markers, maternity and paternity testing confirmed that in all three cases the alleles present were indeed of paternal origin (data not shown). *b*, Gene copy number determination for patients BW11P, BW15P, BW21P and control individuals: Southern blot experiments. Top, 11p15 probes, *INS*, *IGF2*, *HBB*, and *HBBP*; bottom, the same filter was hybridized with a non-11p probe, *COL1A1*, used as the internal control. Lanes 1 and 4, control individuals; lane 2, BW15P; lane 3, BW21P and lane 5, BW11P. All three patients displayed two copies for each of the markers tested. *c*, Densitometry data. Densitometer scanning profiles for *IGF2* (A) and *COL1A1* (B). *d*, Densitometry values obtained by scanning of the blots shown in *b*. *R*, Ratio obtained by dividing the intensity for the 11p15 marker by the intensity of the internal control (*COL1A1*); *P/C*, ratio obtained by dividing the preceding ratio for the patient by that of the control individuals; *C*, mean value for control individuals in lane 1 and 4. METHODS. Methods and probes were as described in Table 1. The same filters were successively hybridized with probes for which the patients were disomic and with a non-chromosome 11 probe (collagen type I alpha 1, *COL1A1*, on chromosome 17). The intensity of the hybridization signals was measured with a SEBIA densitometer. The values for normal control subjects and for patients were estimated through three independent determinations. The value of the 11p15.5 probe versus non-11 internal control probe ratio was calculated for each independent determination. The patient versus control ratio (*P/C*) is indicated for each patient showing paternal disomy. For each patient, the *P/C* ratio was not very different from 1 (which means that there are two copies of the markers in the DNA of the control and in the DNA of the patient).



| d     | INS | COL1A1 | $R = \frac{INS}{COL1A1}$ | $\frac{P}{C}$ | IGF2 | COL1A1 | $R = \frac{IGF2}{COL1A1}$ | $\frac{P}{C}$ | HBB | COL1A1 | $R = \frac{HBB}{COL1A1}$ | $\frac{P}{C}$ | HBBP | COL1A1 | $R = \frac{HBBP}{COL1A1}$ | $\frac{P}{C}$ |
|-------|-----|--------|--------------------------|---------------|------|--------|---------------------------|---------------|-----|--------|--------------------------|---------------|------|--------|---------------------------|---------------|
| C     | 57  | 235    | 0.24                     | —             | 497  | 234    | 2.12                      | —             | 367 | 217    | 1.69                     | —             | 369  | 43     | 8.58                      | —             |
| BW15P | 28  | 131    | 0.21                     | 0.87          | 294  | 130    | 2.26                      | 1.06          | 326 | 190    | 1.71                     | 1.01          | 461  | 64     | 7.20                      | 0.83          |
| BW21P | 35  | 122    | 0.28                     | 1.16          | 252  | 122    | 2.06                      | 0.97          | 167 | 81     | 2.06                     | 1.21          | 169  | 20     | 8.45                      | 0.98          |
| BW11P | 65  | 258    | 0.25                     | 1.04          | 569  | 238    | 2.39                      | 1.12          | 223 | 115    | 1.93                     | 1.14          | 454  | 45     | 10.08                     | 1.17          |



TABLE 2 Genotypes of 21 sporadic BWS patients for nine 11p15.5 RFLPs

|               | HRAS1<br><i>Bam</i> HI | TH<br><i>Taq</i> I | INS<br><i>Rsa</i> I | INS<br><i>Hind</i> III | IGF2<br><i>Sac</i> I | IGF2<br><i>Av</i> II | D11S12<br><i>Msp</i> I | HBB<br><i>Av</i> II | HBBP<br><i>Hinc</i> II |
|---------------|------------------------|--------------------|---------------------|------------------------|----------------------|----------------------|------------------------|---------------------|------------------------|
| BW1P          | 1/1                    | 2/2                | 2/2                 | 1/1                    | 1/2                  | 1/2                  | 2/2                    | 1/2                 | 2/3                    |
| BW2P          | 1/1                    | 2/2                | 2/2                 | 1/2                    | 1/2                  | 1/2                  | 2/2                    | 1/2                 | 2/3                    |
| BW3P          | 1/2                    | 2/2                | 2/2                 | 2/2                    | 2/2                  | 1/1                  | 1/2                    | 2/2                 | 1/1                    |
| BW4P          | 1/2                    | 2/2                | 2/2                 | 2/2                    | 2/2                  | 1/1                  | 2/2                    | 2/2                 | 1/1                    |
| BW5P          | 1/1                    | 2/2                | 1/2                 | 2/2                    | 2/2                  | 1/1                  | 2/2                    | 2/2                 | 1/1                    |
| BW6P          | 1/2                    | 2/2                | 2/2                 | 2/2                    | 2/2                  | 1/1                  | 1/2                    | 1/2                 | 1/1                    |
| BW7P          | 1/2                    | 2/2                | 2/2                 | 1/1                    | 2/2                  | 1/1                  | 2/2                    | 2/2                 | 1/1                    |
| BW8P          | 2/2                    | 2/2                | 1/1                 | 1/1                    | 1/2                  | 1/2                  | 1/2                    | 2/2                 | 1/2                    |
| BW9P          | 1/2                    | 1/2                | 2/2                 | 2/2                    | 2/2                  | 1/1                  | 1/2                    | 1/2                 | 1/1                    |
| BW10P         | 1/2                    | 2/2                | 1/1                 | 1/1                    | 1/2                  | 1/1                  | 2/2                    | 1/2                 | 1/1                    |
| BW11P         | 2/2                    | 2/2                | 1/1                 | 1/1                    | 2/2                  | 1/1                  | 2/2                    | 2/2                 | 1/1                    |
| BW12P         | 2/2                    | 2/2                | 1/1                 | 1/2                    | 1/1                  | 2/2                  | 1/2                    | 1/1                 | 2/2                    |
| BW13P         | 1/2                    | 2/2                | 1/1                 | 1/2                    | 2/2                  | 1/1                  | 1/2                    | 2/2                 | 1/2                    |
| BW14P         | 1/2                    | 2/2                | 1/1                 | 2/2                    | 1/2                  | 1/1                  | 1/2                    | 2/2                 | 1/1                    |
| BW15P         | 2/2                    | 1/2                | 2/2                 | 2/2                    | 2/2                  | 1/1                  | 1/1                    | 2/2                 | 1/1                    |
| BW16P         | 1/2                    | 2/2                | 2/2                 | 1/2                    | 1/2                  | 1/2                  | 2/2                    | 1/2                 | 1/3                    |
| BW17P         | 2/2                    | 2/2                | 1/2                 | 1/2                    | 2/2                  | 2/2                  | 2/2                    | 2/2                 | 1/3                    |
| BW18P         | 2/2                    | 2/2                | 1/2                 | 2/2                    | 2/2                  | 1/1                  | 2/2                    | 2/2                 | 1/1                    |
| BW19P         | 2/2                    | 1/2                | 2/2                 | 2/2                    | 2/2                  | 1/1                  | 1/2                    | 2/2                 | 1/3                    |
| BW20P         | 1/2                    | 1/2                | 2/2                 | 2/2                    | 2/2                  | 1/1                  | 1/2                    | 2/2                 | 1/2                    |
| BW21P         | 2/2                    | 2/2                | 1/1                 | 1/1                    | 2/2                  | 1/1                  | 2/2                    | 2/2                 | 2/2                    |
| $F_{BWS}$     | 0.52                   | 0.80               | 0.85                | 0.76                   | 0.71                 | 0.80                 | 0.57                   | 0.66                | 0.57                   |
| $F_C$         | —                      | 0.78<br>(28/36)    | 0.52<br>(19/36)     | 0.51<br>(18/35)        | 0.51<br>(18/35)      | 0.58<br>(21/36)      | —                      | —                   | —                      |
| $F_{C/HGM10}$ | 0.52                   | 0.73               | 0.52                | 0.45                   | 0.55                 | 0.62                 | 0.73                   | 0.68                | 0.51                   |

$F_{BWS}$ , Frequency of homozygous individuals in the BWS population;  $F_C$ , frequency of homozygous individuals in the control population. The numbers in brackets indicate the number of homozygous individuals versus the total number of individuals tested.  $F_{C/HGM10}$ , Frequency of homozygotes as from HGM 10 (ref. 16);  $P$ , probability that the random distribution of the alleles is responsible for the higher homozygosity in patients than in controls. For the four contiguous loci (INS/*Rsa*I, INS/*Hind*III, IGF2/*Sac*I, and IGF2/*Av*II) taken together, the frequency of homozygotes was 0.48 (10/21) for BWS patients and 0.18 (6/34) for controls. Methods and probes were as described in Table 1. The test used is a simple one-tailed  $\chi^2$  homogeneity test (valid since all the expected values are greater than 6);  $\chi^2 = 5.65$ ,  $P < 0.01$ . The one-tailed test was used because in the hypothesis of isodisomy the frequency of homozygotes is expected to be higher than in controls.

syndromes: the Prader-Willi syndrome with a lack of 15q11-q13 paternal alleles<sup>14</sup>, and the Angelman syndrome with a lack of 15q11-q13 maternal alleles<sup>15</sup>. Taken together the lack of maternal alleles in the three sporadic BWS cases reported here, paternal duplication in trisomic BWS patients, retention of paternal alleles in tumours, and higher penetrance in individuals born to female carriers in familial BWS corroborate the involvement of genomic imprinting. Thus we conclude that an unbalanced dosage of maternal and paternal alleles is a common feature for the different aetiological forms of BWS and associated tumours. □

## Embryological and molecular investigations of parental imprinting on mouse chromosome 7

A. C. Ferguson-Smith, B. M. Cattanach\*, S. C. Barton, C. V. Beechey\* & M. A. Surani

Department of Molecular Embryology, AFRC Institute of Animal Physiology & Genetics Research, Babraham, Cambridge CB2 4AT, UK

\*MRC Radiobiology Unit, Chilton, Didcot, Oxford OX1 0RD, UK

**MOUSE embryos with duplications of whole maternal (parthenogenetic and gynogenetic) or paternal (androgenetic) genomes show reciprocal phenotypes and do not develop to term<sup>1,2</sup>. Genetic complementation has identified the distal region of chromosome 7 (Chr 7) as one of the regions for which both a maternal and paternal chromosome copy are essential for normal development, presumably because of the presence of imprinted genes whose expression is dependent on their parental origin<sup>3,4</sup>. Embryos with the maternal duplication and paternal deficiency of distal Chr 7 are growth retarded and die around day 16 of gestation; the reciprocal paternal duplication embryos die at an unidentified earlier stage<sup>4</sup>. We report here the incorporation of cells with the paternal duplication into chimaeras, resulting in a striking growth enhancement of the embryos. One gene located on mouse distal Chr 7 (ref. 5) is the insulin-like growth factor 2 (*Igf2*) gene, an embryonic mitogen<sup>6</sup>. In embryos with the maternal duplication of distal Chr 7, the two maternal alleles of the *Igf2* gene are repressed. The presence of two paternal alleles of this gene in many cells is probably responsible for the growth enhancement observed in chimaeras. We propose that there are other imprinted genes in**

Received 18 December 1990; accepted 7 May 1991.

1. Turleau, C. & Grouchy, J. de *Ann. Génét.* **28**, 93-96 (1985).
2. Aleck, K., Williams, J., Mongkolkeha, C., Knight, S. & Taysi, K. *Ann. Génét.* **28**, 102-106 (1985).
3. Henry, I. et al. *Hum. Genet.* **81**, 273-277 (1989).
4. Ping, A. J. et al. *Am. J. hum. Genet.* **44**, 720-723 (1989).
5. Koufos, A. et al. *Am. J. hum. Genet.* **44**, 711-719 (1989).
6. Wiedemann, H. R. *Eur. J. Ped.* **141**, 129 (1983).
7. Lubinsky, M., Hermann, J., Kossef, A. L. & Opitz, J. M. *Lancet* **i**, 932 (1974).
8. Schroeder, W. T. et al. *Am. J. hum. Genet.* **40**, 413-420 (1987).
9. Mannens, M. et al. *Hum. Genet.* **31**, 41-48 (1988).
10. Scrabble, H. et al. *Proc. natn. Acad. Sci. U.S.A.* **86**, 7480-7484 (1989).
11. Spence, J. E. et al. *Am. J. hum. Genet.* **42**, 217-226 (1988).
12. Voss, R. et al. *Am. J. hum. Genet.* **45**, 373-380 (1989).
13. Niikawa, N. et al. *Am. J. med. Genet.* **24**, 41-55 (1986).
14. Nicholls, R. D., Knoll, J. H. M., Butler, M. G., Karam, S. & Lalande, M. *Nature* **342**, 281-285 (1989).
15. Knoll, J. H. M. et al. *Am. J. hum. Genet.* **47**, 149-155 (1990).
16. Henry, I. et al. *Ann. hum. Genet.* **49**, 173-180 (1985).
17. Junien, C. & McBride, O. W. *Cytogenet. Cell Genet.* **51**, 226-258 (1989).
18. Koufos, A. et al. *Nature* **309**, 170-172 (1984).
19. Orkin, S. H., Goldman, D. S. & Sallan, S. T. *Nature* **309**, 172-174 (1984).
20. Reeve, A. E. et al. *Nature* **309**, 174-176 (1984).
21. Fearon, E. R., Vogelstein, B. & Feinberg, A. P. *Nature* **309**, 176-177 (1984).
22. Scrabble, H. J., Witte, D. P., Lampkin, B. C. & Caveness, W. K. *Nature* **329**, 645-647 (1987).
23. Mannens, M. et al. *Cytogenet. Cell Genet.* **46**, 655 (1988).
24. Henry, I. et al. *Proc. natn. Acad. Sci. U.S.A.* **86**, 3247-3251 (1989).
25. Takahiko, Y. et al. *J. natn. Cancer Inst.* **81**, 518-523 (1989).

**ACKNOWLEDGEMENTS.** We thank P. Chardin, J. Bell, M. Goossens, Y. Lebourg, J. Mallet and R. White for probes, E. Azria, M. L. Briard, L. Brugière, J. L. Chaussain, J. de Grouchy, J. P. Harpey, D. Labie, P. Landrieu, H. J. Plenzel, Ribier, J. M. Saudubray and C. Turleau who referred the patients. This work was supported by INSERM, the Ministère de la Recherche et de la Technologie, the Ligue Nationale contre le Cancer and the Association de Recherche Contre le Cancer.

this Chr 7 region. We also compare the imprinting of this sub-genomic region with phenotypes resulting from the duplication of the whole parental genome in parthenogenones and androgenones.

We investigated the developmental potential of cells carrying the paternal duplication of the region distal to the T(7B3;15C)9H translocation breakpoint on Chr 7 (ref. 7) in chimaeras. Blastocysts carrying the paternal duplication of distal Chr 7 (PatDi7) were expected at a frequency of about 17% (ref. 8). Two markers were used to identify the experimental cells: glucose phosphate isomerase-1 (*Gpi-1*), and albino (*c*) which is located on distal Chr 7. Inner-cell masses (ICMs) were released from putative PatDi7 blastocysts and injected into

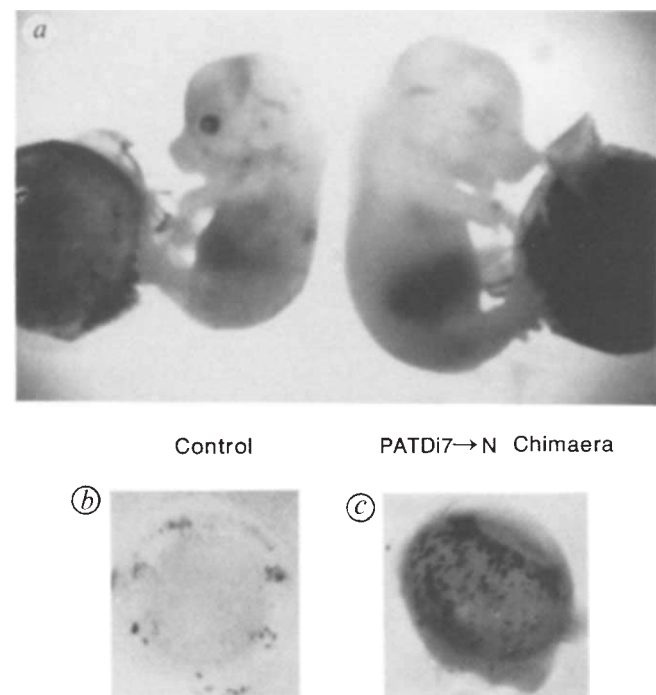


FIG. 1 *a*, Comparison of (e15) PatDi7→N chimaera (right) embryo and control-manipulated but nonchimaeric littermate (left) showing growth enhancement in the chimaera due to the presence of cells carrying the paternal duplication of the distal region of Chr 7. The chimaera was about 50% larger by weight than controls. *b*, Eye from e15 PatDi7→N chimaera in *a*. The pigmented cells are derived from the host component and the *c/c* cells from the donor PatDi7 ICM. The amount of pigment reflects the extent of chimaerism as assayed by GPI-1 isozyme analysis. *c*, Eye from e17 PatDi7→N chimaera with pigmented and nonpigmented components as described in *b*. This embryo was about 55% larger by weight than the controls. METHODS. Construction of chimaeras: e4 blastocysts (*Gpi-1<sup>a</sup>/Gpi-1<sup>a</sup>*) were isolated from uteri of T9H/++♀×T9Hc/+♂ intercrosses and cultured *in vitro* for 2 days<sup>23</sup>. These blastocysts included the genetically marked and balanced embryos carrying the paternal duplication occurring at a frequency of about 17%. Many of the embryos from the intercross were arrested around the four-cell stage due to the many genetically unbalanced gametes produced. This further reduced the number of blastocysts which provided donor ICMs. ICMs were isolated by immunosurgery<sup>24</sup> and microinjected into expanded normal host blastocysts (CBA×C57B1/6J)<sub>F<sub>1</sub></sub> (*Gpi-1<sup>b</sup>/Gpi-1<sup>b</sup>*) using methods described previously<sup>23,25,26</sup> with the following modifications (S.C.B. & R. Fundele, unpublished results); injection pipettes were bevelled, with an internal diameter of 22 µm and ICMs were briefly stored in medium supplemented with cytochalasin B (5 µg ml<sup>-1</sup>) and Nocodazole (1.5 µg ml<sup>-1</sup>) to allow them to be distorted without damage to the cells. The manipulated embryos were then transferred into the uteri of pseudopregnant recipients 3 days *post coitum*. Embryos were removed at stages e15–e17.5, dissected and analysed for GPI-1 isozyme type for chimaerism<sup>23</sup>. Chimaeric embryos were GPI-1A+GPI-1B type and in the PatDi7→N chimaeras also had albinism in the eye indicative of two copies of paternal *c* alleles in the pigment cells derived from the donor ICM. Growth enhancement always correlated with albinism in the eye.

normal host blastocysts (N) to generate chimaeras. Three PatDi7→N chimaeras were obtained and exhibited a striking phenotype; a substantial size increase (Fig. 1*a* and Table 1). For an e15 PatDi7→N embryo the increase was 51% with the PatDi7 cells present at about 75% in 10 tissues analysed (Fig. 1*b* and Table 1). An e15.5 PatDi7→N embryo was 35% larger than controls with 50% contribution of PatDi7 cells in limbs which were the only tissues assayed for GPI; the remaining embryo was used for northern blot analysis. The 17-day-old PatDi7→N embryo was 55% larger with nearly equal contributions to the 14 tissues analysed (Fig. 1*c* and Table 1). These sizes were compared with both manipulated and nonmanipulated embryos carrying normal ICMs. The results demonstrate that the PatDi7 cells were 'rescuable' in chimaeras and that a large contribution from PatDi7 cells was responsible for enhanced growth.

The maternal duplication of distal Chr 7 produces a less severe phenotype with embryos dying around day 16. ICMs from blastocysts, with the maternal duplication of distal Chr 7 (MatDi7), also expected at the low frequency of about 17% (ref. 8) were introduced into normal host blastocysts. These embryos were allowed to develop to term. Two of these were MatDi7→N chimaeras, as determined by coat colour and GPI-1 analysis. The contribution of MatDi7 cells was approximately 25–67% in all 28 tissues analysed in one 2-week-old mouse and in 18 tissues analysed in a 6-month-old mouse without major differences in the relative tissue distributions between the two animals. Importantly, the body weights of the MatDi7→N chimaeras at or after birth did not differ significantly from those of the chimaeric or nonchimaeric controls (see legend to Fig. 2).

To identify imprinted genes on distal Chr 7, nonchimaeric MatDi7 embryos, which can develop to day 16 were used because, although these are roughly 50% smaller than controls<sup>4</sup> (Fig. 2*a*), there were no obvious histological malformations (not shown). Expression of genes which map to distal Chr 7 was investigated by northern blot analysis. One of these genes is *Igf2* (ref. 5) which is an embryonal mitogen<sup>6</sup> widely expressed in several embryonic and extraembryonic tissues in the mouse<sup>9</sup>. The expression of *Igf2* was assayed because of the growth phenotypes described here. *Igf2* expression is virtually undetectable in the e13–e15 MatDi7 embryos and placentae compared with controls (Fig. 2*b*), although low expression from maternal alleles is not excluded which may be confined to a subset of cells (see ref. 10). The e15.5 PatDi7→N embryo chimaera also showed an increase in *Igf2* expression of about 31% as assayed by a series of densitometric scans of northern blots (not shown). In the e15 hairpin-tail mutant embryos (T<sup>hp</sup>), which were used as controls because, like the MatDi7 embryos, they also die at day 16 (see legend to Fig. 2), *Igf2* expression was normal (Fig. 2*b*). These data are consistent with RNase protection data derived from targeted mutagenesis of *Igf2* (ref. 10). The *Igf2* receptor gene is also imprinted but with the maternal allele being active and the paternal allele being inactive<sup>11</sup>; the opposite of the ligand. These findings suggest a critical role for these imprinted genes in controlling normal mammalian development.

It is of interest to compare these chimaeric phenotypes with those containing either androgenetic (AG) or parthenogenetic (PG) cells in the presence of normal cells (N). The AG→N chimaeras showed a comparable size increase to that of the PatDi7→N chimaeras<sup>12</sup> whereas the PG→N chimaeras were smaller by a similar proportion<sup>13</sup>. Indeed preliminary results show that *Igf2* expression is substantially higher in AG than in PG conceptuses (R. Ohlsson, A. Glaser, A.F.-S., S.C.B. and M.A.S., manuscript in preparation). The size of MatDi7→N chimaeras was comparable to the controls, which could be attributed to the overall dosage of *Igf2* reaching a threshold in the presence of normal cells. This is consistent with the proposed autocrine-paracrine action of *Igf2*<sup>6,14</sup>. Hence, the growth retardation in PG→N chimaeras is probably due to the cumulative effects of other imprinted genes in PG cells involved in



growth regulation. The distribution of AG and PG cells in chimaeras is non-uniform and reciprocal, unlike that of the MatDi7 and PatDi7 cells. AG cells contribute disproportionately to tissues of mesodermal origin such as the skeletal muscle, heart and kidney and to ectodermally derived tissues such as the brain and epidermis<sup>12,15</sup>. The contribution of PG cells in chimaeras is most consistently in the brain, epidermis and germ

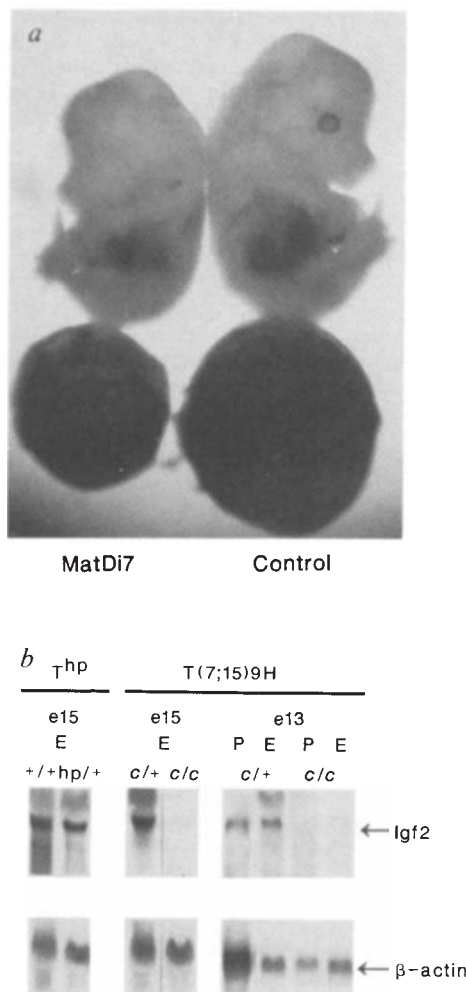


FIG. 2 *a* Comparison of e15 MatDi7 (left) and control embryo and placenta derived from the T9Hc/+c $\times$  T9H+/++ $\delta$  intercross<sup>4</sup> illustrating the growth retardation of both the embryo and placenta in the presence of two maternal copies of the distal region of Chr 7. The MatDi7 embryo lacks pigment in the eye due to the presence of the two maternal *c* alleles. Weights of two liveborn MatDi7  $\rightarrow$  N chimaeras were 5.0 g and 4.0 g compared with 5.7 g and 4.1 g, respectively, of concurrent controls indicating no significant size difference. *b*, Igf2 expression analysis of experimental and control e13 and e15 embryos (E) and placentae (P). T9H RNA was from T9Hc/+c $\times$  T9H+/++ $\delta$ . *c/c*, Maternal duplication for distal Chr 7; *c/+*, nonalbino littermates (see *a*). T<sup>hp</sup> RNA was from T<sup>hp</sup> ins/+ (CBA  $\times$  C57B1/6J)F<sub>1</sub> $\delta$   $\times$  (CBA  $\times$  C56B1/6J)F<sub>1</sub> $\delta$ <sup>27</sup>. In this intercross, maternal inheritance of the mutation results in a mutant phenotype whereas the reciprocal intercross, where the mutation is paternally inherited, does not<sup>11,27</sup>. Mutant embryos were distinguished from normal littermates by their mutant tails and the unambiguous presence of oedema. +/+, Control littermate; hp/+, maternally inherited hairpin-tail mutant embryos. The Igf2 probe is derived from exon 6 (ref. 5) hybridizing to a major transcript of ~4.8 kilobases.  $\beta$ -actin is shown as a qualitative and quantitative control and illustrates that actin is transcribed in the absence of Igf2 in both the *c/c* embryo and placenta. METHODS. Embryos were killed at e13–e15 and RNA was isolated separately from embryos and placentae using methods described previously<sup>26</sup>. RNA (10–20  $\mu$ g) was separated on formaldehyde gels, transferred to nylon membranes and hybridized as shown with <sup>32</sup>P-radiolabelled probes<sup>29</sup>.

TABLE 1 Analysis of embryos

| Embryo age (days) | No. of litters | Embryo                 | n  | GPI   | Mean weight $\pm$ s.d. E(mg) | P(mg)        |
|-------------------|----------------|------------------------|----|-------|------------------------------|--------------|
| e15               | 1              | Unoperated             | 2  | B     | 258 $\pm$ 44*                | 89 $\pm$ 12  |
|                   |                | Unchimaeric            | 2  | B     | 230 $\pm$ 18*                | 81 $\pm$ 5   |
|                   |                | PatDi7 $\rightarrow$ N | 1  | A + B | 369 (51%)                    | 117          |
| e15.5             | 2              | Unoperated             | 5  | B     | 348 $\pm$ 32                 | 99 $\pm$ 12  |
|                   |                | Unchimaeric            | 4  | B     | 326 $\pm$ 13                 | 81 $\pm$ 12  |
|                   |                | Chimaeric              | 1  | A + B | 329                          | 99           |
|                   |                | PatDi7 $\rightarrow$ N | 1  | A + B | 450 (35%)                    | 104          |
| e16               | 4              | Unoperated             | 12 | B     | 402 $\pm$ 68                 | 98 $\pm$ 12  |
|                   |                | Unchimaeric            | 10 | B     | 398 $\pm$ 42                 | 94 $\pm$ 15  |
|                   |                | Chimaeric              | 1  | A + B | 406                          | 113          |
| e17               | 5              | Operated               | 11 | B     | 615 $\pm$ 79                 | 108 $\pm$ 10 |
|                   |                | Unchimaeric            | 14 | B     | 522 $\pm$ 77                 | 116 $\pm$ 27 |
|                   |                | Chimaeric              | 9  | A + B | 599 $\pm$ 88                 | 104 $\pm$ 20 |
|                   |                | PatDi7 $\rightarrow$ N | 1  | A + B | 869 (55%)                    | 111          |
| e17.5             | 2              | Unchimaeric            | 5  | B     | 640 $\pm$ 122                | 109 $\pm$ 14 |
|                   |                | Chimaeric              | 4  | A + B | 791 $\pm$ 174                | 113 $\pm$ 15 |

A total of 83 embryos were recovered of 117 transferred to pseudopregnant recipients; 53 manipulated embryos of 80 transferred were recovered; 3 recipients failed to become pregnant. e1 is the day of vaginal-plug appearance. E, Embryo; P, placenta; s.d., standard deviation for  $n > 2$ ;  $n$ , number of embryos recovered; N, normal fertilized component of chimaera. The percentage in parenthesis indicates the growth enhancement of the PatDi7  $\rightarrow$  N chimaera compared with all concurrent controls. GPI, the isozyme type denoted A and/or B. The following 14 tissues were dissected from each manipulated embryo and assayed for chimaerism by GPI-1 typing: brain, eyes, tongue, heart, lung, thymus, forelimb, hindlimb, gut, pancreas, liver, kidneys, gonads. For some embryos placenta, blood and skin were included. Chimaeras contained roughly uniform contributions of experimental and host cells to all tissues except for placenta and blood where there were very few experimental cells. This is expected for placenta which is predominantly host-derived. Only the limbs of the e15.5 PatDi7  $\rightarrow$  N were assayed for GPI-1.

\* These values are means  $\pm$  difference.

cells<sup>12,13,16</sup>. By contrast, MatDi7 cells are detected in skeletal muscle to which PG cells rarely make a contribution, presumably due to the action of other imprinted genes in PG cells.

We believe that imprinted genes in addition to *Igf2* are present on distal Chr 7. First, targeted mutagenesis of *Igf2* followed by the transmission of the intact maternal allele resulted in mice that, although growth retarded, were live and fertile<sup>17</sup> whereas the MatDi7 embryos die at around day 16. Second, duplications of a region more distal to the T(7;15)9H breakpoint may also produce embryos of a less severe phenotype<sup>4</sup>. The early demise of the unchimaeric PatDi7 embryos may be a result of two active paternal alleles of *Igf2* or due to the action of other imprinted genes.

Human Chr 11p15, which shares syntenic homology with mouse distal Chr 7, may also be imprinted. The paternal duplication of the region seems to be associated with enhanced growth dysmorphologies linked to the Beckwith–Wiedemann syndrome and also to embryonal tumours of mesodermally derived tissues including rhabdomyosarcoma and Wilms' tumour<sup>18–20</sup>. The expression of *Igf2* is elevated in these tumours as well as tumours of the trophoblast<sup>21</sup>, a site of normal *Igf2* expression. A cloned sequence with homology to the imprinted region of human Chr 15 associated with the Prader–Willi/Angelman syndromes has been mapped to mouse Chr 7<sup>22</sup>. Detailed investigations in the mouse, particularly of the paternal duplication of distal Chr 7 as seen in the enhanced growth of PatDi7  $\rightarrow$  N embryos, may aid our understanding of the genesis of these human genetic disorders.

We propose that this parental duplication–deficiency system provides a unique and powerful model to investigate the mechanism of imprinting. This is because both imprinted alleles of a gene are of the same parental origin and therefore likely

to have the same molecular characteristics, unlike in normal cells where the two parental alleles will have a different imprint. We have thus begun a detailed analysis of this chromosomal region using cells carrying the maternal duplication. These studies could provide an understanding of the chromatin structure and DNA modifications responsible for the differential parental gene activities which underlie chromosomal imprinting. *Note added in proof.* While this paper was in press an additional gene on distal Chr 7, H19, was shown to be imprinted<sup>30</sup> supporting our proposal that there is at least one other imprinted gene in this region. □

Received 25 February; accepted 26 April 1991.

1. Surani, M. A. H. in *Experimental Approaches to Mammalian Embryonic Development* (ed. Rossant, J. & Pedersen, R. A.) 401–435. (Cambridge University Press, Cambridge, 1986).
2. Solter, D. A. *Rev. Genet.* **22**, 127–146 (1988).
3. Cattanach, B. M. *J. Embryol. exp. Morph.* **97**, 137–150 (1986).
4. Searle, A. G. & Beechey, C. V. *Genet. Res.* **56**, 237–244 (1990).
5. Rotwein, P. & Hall, L. *DNA Cell Biol.* **9**, 725–735 (1990).
6. Beck, F. *et al. Development* **101**, 175–184 (1987).
7. Lyon, M. F. & A. G. Searle, A. G. *Genetic Variants and Strains of the Laboratory Mouse* 2nd edn 592 (Oxford University Press, Oxford, 1989).
8. Searle, A. G., Ford, C. E. & Beechey, C. V. *Genet. Res.* **18**, 215–235 (1971).
9. Lee, J., Pintar, J. & Efstratiadis, A. *Development* **110**, 151–159 (1990).
10. De Chiara, T. M., Robertson, E. J. & Efstratiadis, A. *Cell* **64**, 849–860 (1991).
11. Barlow, D. P., Stoger, R., Herrmann, B. G., Saito, K. & Schweifer, N. *Nature* **349**, 84–87 (1991).
12. Surani, M. A. *et al. Development Suppl.* 89–98 (1990).
13. Fundele, R. *et al. Development* **108**, 203–211 (1990).
14. Stylianopoulou, F. *et al. Development* **103**, 497–506 (1988).
15. Mann, J. R., Gadi, I., Harbison, M. L., Abbondanzo, S. J. & Stewart, C. L. *Cell* **62**, 251–260 (1990).
16. Fundele, R., Norris, M., Barton, S. C., Reik, W. & Surani, M. A. *Development* **106**, 28–35 (1989).
17. De Chiara, T. M., Efstratiadis, A. & Robertson, E. J. *Nature* **345**, 78–80 (1990).
18. Reik, W. *Trends Genet.* **5**, 331–336 (1989).
19. Hall, J. *Am. J. hum. Genet.* **46**, 857–873 (1990).
20. Ferguson-Smith, A. C., Reik, W. & Surani, M. A. *Cancer Surveys* **9**, 487–503 (1990).
21. Ohlsson, R., Holmgren, L., Glaser, A., Szpecht, A. & Pfeifer-Ohlsson, S. *EMBO J.* **8**, 1993–1999 (1989).
22. Nicholls, R. *et al. Am. J. hum. Genet.* **46**, A230 (1990).
23. Barton, S. C., Adams, C. A., Norris, M. L. & Surani, M. A. *J. Embryol. exp. Morph.* **90**, 267–285 (1985).
24. Solter, D. & Knowles, B. *Proc. natn. Acad. Sci. U.S.A.* **72**, 5099–5102 (1975).
25. Babinet, C. *Exp. Cell Res.* **130**, 15–19 (1980).
26. Gardner, R. L. in *Methods in Mammalian Reproduction* (ed. Daniel, J. C. Jr) 137–165 (Academic Press, New York, New York, 1978).
27. Winking, H. & Silver, L. M. *Genetics* **108**, 1013–1020 (1984).
28. Chomcynski, P. & Sacchi, N. *Ann. Biochem.* **162**, 156–159 (1987).
29. Maniatis, T., Fritsch, E. F. & Sambrook, J. *Molecular Cloning: a Laboratory Manual* (CSH, USA, 1982).
30. Bartolomei, M. S., Zemel, S. & Tilghman, S. M. *Nature* **351**, 153–155 (1991).

ACKNOWLEDGEMENTS. We acknowledge the earlier work of A. G. Searle in establishing this translocation in the mouse. We thank W. Reik, R. Fundele, P. Jones, B. Newman and H. Sasaki for help and advice with this study. W. Mills and M. Davis for technical assistance, P. Rotwein for the Igf2 cosmid and R. Kothary for the actin clone. A.F.-S. is supported by a Babraham Research Fellowship.

## Sulphated lipo-oligosaccharide signals of *Rhizobium meliloti* elicit root nodule organogenesis in alfalfa

Georges Truchet, Philippe Roche\*, Patrice Lerouge\*, Jacques Vasse, Sylvie Camut, Françoise de Billy, Jean-Claude Promé\* & Jean Dénarié

Laboratoire de Biologie Moléculaire des Relations Plantes-Microorganismes, CNRS-INRA, BP 27, 31326 Castanet-Tolosan Cedex, France

\* Centre de Recherches de Biochimie et de Génétique Cellulaire, CNRS LP8201, 118 Route de Narbonne, 31062 Toulouse Cedex, France

*RHIZOBIUM meliloti* is a symbiotic bacterium that elicits the morphogenesis of nitrogen-fixing nodules, specific organs on the roots of alfalfa (*Medicago sativa*)<sup>1</sup>. In *R. meliloti* a series of nodulation (*nod*) genes have been identified which are involved in root-hair curling and infection and in nodule formation<sup>1</sup>. The *nodABC* genes, common to all *Rhizobium* sp., and the host-range *nodH* and *nodPQ* genes are involved in the production of an excreted root-hair deforming factor, NodRm-1, which is a sulphated and acylated glucosamine oligosaccharide<sup>2–7</sup>. Here we

report that purified NodRm-1 and a related compound, Ac-NodRm-1, at concentrations in the micromolar–nanomolar range, elicit cortical cell divisions and the formation of genuine nodules on aseptically grown seedlings of alfalfa. Chemical modifications of NodRm-1, such as the removal of the sulphate group, reduction of the terminal sugar or hydrogenation of the acyl chain result in a strong decrease in the morphogenic activity. A highly specific prokaryotic lipo-oligosaccharide signal is thus able to trigger a genuine organogenesis in a higher plant.

The early steps in the development of the alfalfa–*R. meliloti* symbiosis involve initiation by the bacterium of the curling of host root hairs and the triggering of cortical cell division leading to nodule organogenesis<sup>1</sup>. Mutations in the *R. meliloti nodABC* genes abolish all these processes and also prevent the production of the NodRm-1 factor<sup>1,4,8</sup>. The *nodABC* operon is expressed in the infection threads close to which cortical cells are dividing (Fig. 1a). Thus NodRm-1 might be involved in the induction not only of root hair deformation but also of cortical cell divisions.

NodRm-1 is obtained from the culture supernatant of *R. meliloti* strains that have been genetically engineered to overproduce the signal<sup>4</sup>. A related factor, Ac-NodRm-1, which is *O*-acetylated at carbon 6 of the terminal non-reducing sugar (Fig. 2), is also found in amounts that vary with each culture<sup>7</sup>. As NodRm-1 and Ac-NodRm-1 are difficult to separate completely, the *O*-acetyl group<sup>7</sup> was removed in order to use a defined, purified molecule, NodRm-1, in the following experiments.

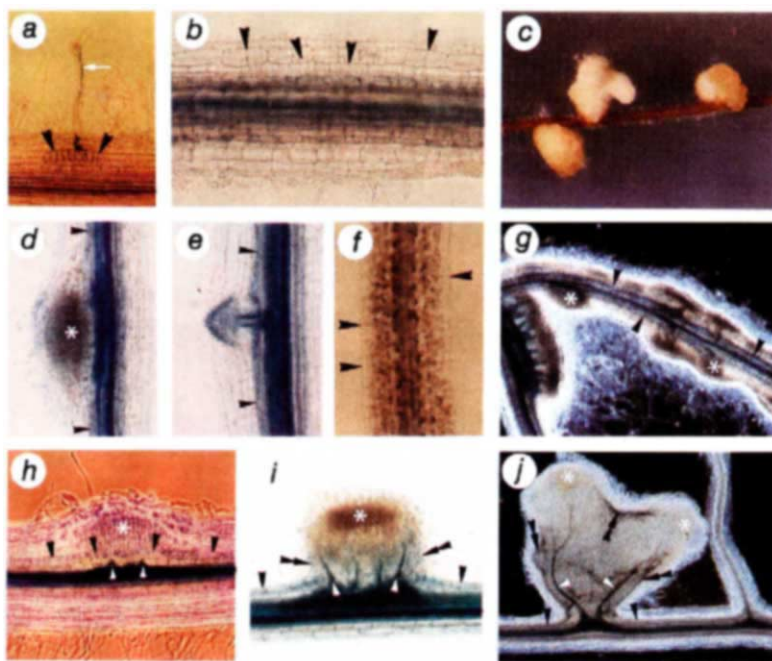
Microscopy studies of the root system of alfalfa seedlings grown for 6, 9 or 12 days in the presence of NodRm-1 showed that at a concentration of 10<sup>−9</sup> M, NodRm-1 induced discrete foci of cell divisions in the inner cortex. No such foci could be observed for untreated control seedlings. At a higher concentration (10<sup>−7</sup>) cell divisions either occurred as discrete foci (Fig. 1b) or extended to larger zones of the root including the whole cortex (Fig. 1f, g).

Alfalfa seedlings were grown aseptically for 4 weeks in the presence of various concentrations of NodRm-1. Root deformations were observed which were distinguishable from the cone-shaped protuberances of lateral roots, and varied in appearance from bumps to round, elongated or multilobate structures (Fig. 1c). Detailed microscopical studies defined their ontogeny, anatomy and histology, to determine on the basis of defined criteria<sup>8,26</sup> whether they are nodules, roots or intermediary structures. Light microscopy showed that these deformations had an early developmental pattern characteristic of rhizobia-induced nodules. The meristems originated from the activity of foci of dividing cortical cells (Fig. 1d, g). In emerging deformations, early cortical cell divisions were followed by an activation of the underlying endodermis and vascular bundles (Fig. 1h). The newly formed bundles developed from at least two, but often four, distinct sites close to the root vascular system (Fig. 1h–j). Later, most of the deformations protruding from the root surface had anatomical and histological features of genuine rhizobia-induced nodules, such as apical meristems and peripheral vascular bundles and endodermis (Fig. 1i, j). These anatomical characteristics were fundamentally different to those of equally-sized developing lateral roots which exhibited differentiation of a single central vasculature surrounded by endodermis (compare Fig. 1d and e, and the root deformation and the lateral root in Fig. 1j). The NodRm-1-induced nodules were bacteria-free because (1) we could not detect colony-forming units when the crushed root-systems of NodRm-1-treated plants were streaked on agar plates; and (2) we could not detect any bacteria by light and electron microscopy of these structures (data not shown). Histological studies of all such root deformations showed that more than 99% of the structures scored on morphological criteria (a nonconical shape) were genuine nodules. We therefore used visual scoring to assay nodule formation in the quantitative experiments.



FIG. 1 Light micrographs of alfalfa roots and developing nodules. *a*, Plant inoculated with a *R. meliloti* strain containing a fusion between the promoter of the *nodABC* genes and the reporter *Escherichia coli lacZ* gene. The fusion expresses the *nodABC* genes in the infection threads in a root hair and in the outer cortex. Note the cell divisions in the inner cortex in front of the infection thread. *b–j*, Plants treated with sterile  $10^{-7}$  M NodRm-1 in the absence of bacteria. *b*, Discrete foci of dividing cells in the cortex of a 9-day-old seedling. *c*, General view of root deformations on a 4-week-old plant. *d*, Emerging nodule of cortical origin. *e*, Emerging secondary root of pericycle origin. *f–g*, Extension of cortical cell divisions to a large area of a secondary root. *h–j*, Nodules at different steps of development: peripheral differentiation of the nodule vasculature and endodermis. Note on *j* a lateral root on the right. White arrow: infection thread; large black arrowheads: cortical cell division; asterisks: nodule meristems; small black arrowheads: root endodermis; black double arrowheads: peripheral nodule endodermis; white arrowheads: nodule vascular bundles.

**METHODS.** Seeds of *M. sativa* cv. Gemini were surface-sterilized and germinated as previously described<sup>26</sup>. Germinating seedlings were aseptically transferred onto agar slants (two seedlings per tube) with nitrogen-free Fåhræus medium. *a*, Three-day-old plants were inoculated with *R. meliloti* 2011(pGMI149*nodA-lacZ*X49), merodiploid for the *nod* region and having an *E. coli lacZ* gene fusion to the *nodABC* promoter<sup>27</sup>.  $\beta$ -Galactosidase activity *in planta* was monitored as previously described<sup>28</sup>. *b–j*, The biological effects of NodRm-1 were assayed by growing plants on an agar medium to which purified and filter-sterilized NodRm-1 was added before pouring (at about 50 °C), at final concentrations ranging from  $10^{-7}$  to  $10^{-10}$  M. Histological observations were made on whole undissected roots. Entire roots collected at regular



intervals were cleared with sodium hypochlorite<sup>26</sup>, stained with methylene blue (0.005% in water) and observed by bright-field, dark-field or phase-contrast microscopy using a Vanox Olympus light microscope. Ultrastructural studies were performed on sections of glutaraldehyde-fixed root deformations processed as already described<sup>13</sup>.

The number of nodules produced over 4 weeks increased as a function of NodRm-1 concentration (Fig. 3*a*). At  $10^{-10}$  M the degree of nodulation was the same as the control in which around 5% of untreated alfalfa seedlings exhibited a spontaneous nodulation, as already reported<sup>9</sup>. At higher concentrations, the addition of NodRm-1 resulted in a more abundant nodulation (Fig. 3*a*) which affected a much higher proportion

of plants, up to 90% at  $10^{-7}$  M (data not shown). The increase was proportional to the concentration. The addition of an excess of combined nitrogen has been reported to suppress nodulation in various legumes, including alfalfa<sup>10</sup>. The addition of 15 mM potassium nitrate resulted in the complete suppression of the induction of nodule formation by  $10^{-7}$  M NodRm-1. Thus the treatment of alfalfa seedling roots by NodRm-1 at low

FIG. 2 Structure of NodRm-1, Ac-NodRm-1 and of the three chemically modified derivatives of NodRm-1, the reduced form (red)NodRm-1, the hydrogenated form (hyd)NodRm-1 and the desulphated form (des)NodRm-1. X and Y correspond to the unmodified parts of NodRm-1.

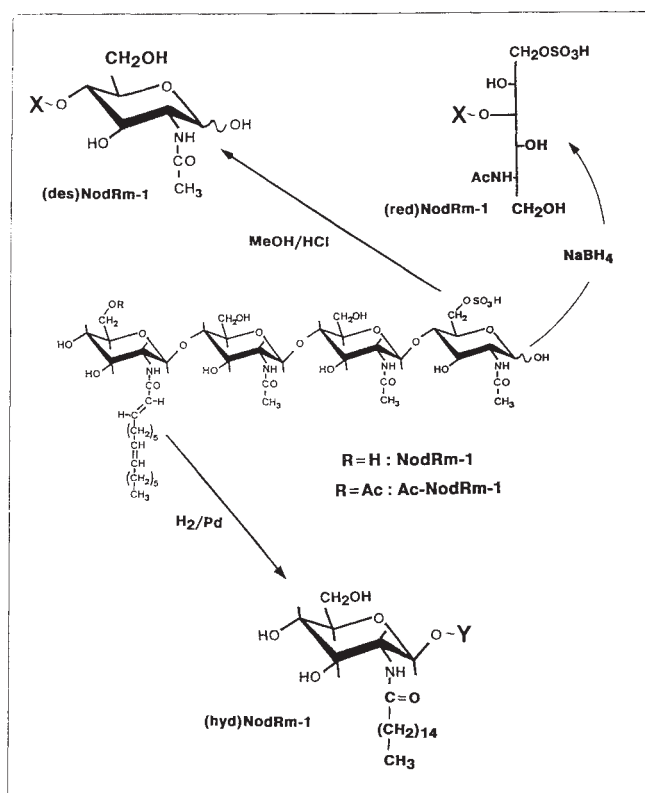
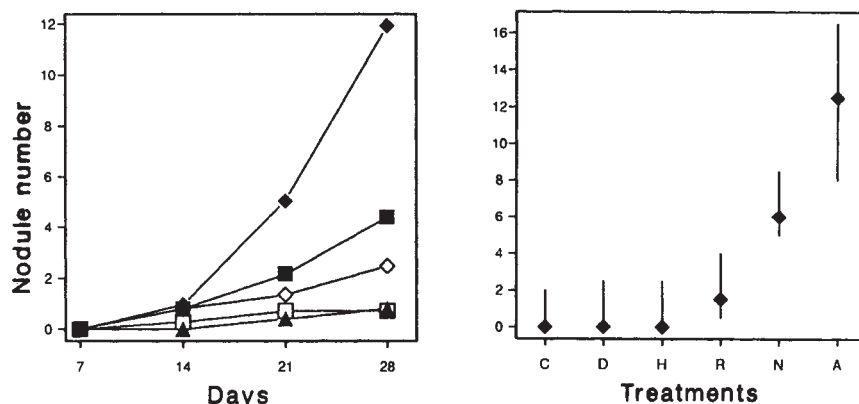


FIG. 3 Induction of nodule formation on alfalfa roots by purified NodRm factors. Nodule numbers are given per two plants. *a*, Number of nodules formed as a function of time at various concentrations of NodRm-1. □, control; ▲,  $10^{-10}$  M; ◇,  $10^{-9}$  M; ■,  $10^{-8}$  M; ◆,  $10^{-7}$  M. *b*, Number of nodules formed in the presence of NodRm-1 and of its chemically modified NodRm-1 derivatives at  $10^{-7}$  M. Points represent the nodule number medians and the vertical bars represent the confidence intervals at the 5% probability level. C: Control; D: (des)NodRm-1; H: (hyd)NodRm-1; R: (red)NodRm-1; N: NodRm-1; A: mixture of Ac-NodRm-1/NodRm-1.

**METHODS.** NodRm-1 and the mixture Ac-NodRm-1/NodRm-1 were isolated from cultures of the overproducing *R. meliloti* strain EJ355(pGM1149) as previously described<sup>7</sup>. The preparation of (red)NodRm-1, (hyd)NodRm-1 and (des)NodRm-1 is described in the legend to Fig. 4. *M. sativa* cv. Gemini seedlings were aseptically grown for 4 weeks on agar plates (five seedlings per 15-cm-diameter Petri dish) (*a*) or on agar slants (two seedlings per tube) (*b*) under the conditions described in the legend to Fig. 1. The number of putative nodules was determined by visually scoring root deformations which did not display the conical shape of developing secondary roots. This gave an underestimation of the biological activity since the meristems not protruding from the root surface (Fig. 2*b*) were not counted; and also the series of small bumps which appeared only after clearing (see Fig. 2*g*) but were not discernible by eye, were scored as only one deformation. To study the anatomy and histology of the root deforma-



tions, entire roots of 4-week-old plants grown in the presence of  $10^{-7}$  M NodRm-1 and NodRm-1/Ac-NodRm-1 were cleared, stained and examined by light microscopy as described in the legend to Fig. 1. Twenty-five and 16 plants per treatment were assayed in experiments *a* and *b*, respectively. Experiments were repeated at least three times; the data shown in this figure are those from two representative single experiments. Sterility of nodules was checked as previously described<sup>9</sup>. In *b*, the median confidence intervals were determined for each treatment using the Wilcoxon signed-rank test<sup>29</sup>. The significance of the differences between the number of nodules elicited by the different compounds was calculated by the Wilcoxon-Mann-Whitney test<sup>29</sup>.

concentrations elicits the formation of genuine nodules which share the ontogenical (a cortical origin), anatomical (peripheral endodermis and vascular bundles) and physiological (nitrate repression of organ formation) features of rhizobia-induced nodules.

To study the relationship between the structure and the activity of NodRm-1, chemically modified molecules were prepared by (1) reduction (red) of the free anomeric carbon of the reducing sugar, (2) hydrogenation (hyd) of the double bonds of the acyl chain or (3) desulphation (des) (see legend to Fig. 4). Fast-atom bombardment mass spectrometry and  $^1\text{H-NMR}$  spectrometry (Fig. 4) were used to verify the structure of the three chemically modified molecules, (red)NodRm-1, (hyd)NodRm-1 and (des)NodRm-1 (Fig. 2), and showed that the chemical treatments did not introduce unwanted modifications. The comparison of the nodulation-inducing ability of NodRm-1 and its modified derivatives at a concentration of  $10^{-7}$  M showed that each modification of NodRm-1 significantly decreased the biological activity (Fig. 3*b*). These results demonstrate that the sulphate group, the reducing function of the sugar backbone and at least one of the two double bonds of the acyl chain are important for triggering alfalfa nodule organogenesis. Moreover, as specific modifications of NodRm-1 alter the biological activity, these results further confirm that the active molecule in the extract is NodRm-1 and not a putative contaminant, such as a compound related to classic phytohormones. Analysis of the structure-function relationships is in progress and involves the chemistry and biological activities (root hair deformation, cortical cell division and nodulation) of the genetically modified NodRm factors which are produced by *R. meliloti* strains mutated in the host-range *nod* genes (P.R. *et al.*, manuscript in preparation).

A mixture of NodRm-1 and Ac-NodRm-1 (around 60:40) was more active than pure NodRm-1 in eliciting nodule formation (Fig. 3*b*). In *R. leguminosarum* bv. *viciae*, lipo-oligosaccharidic Nod signals have recently been identified which are similar to NodRm-1, and *O*-acetylated derivatives have also been found<sup>11</sup>. The *O*-acetylation of the signals seems to require the *nodL* gene<sup>11</sup> whose putative product has homologies with bacterial acetyl transferases<sup>12</sup>. A *nodL* gene has also been identified in *R. meliloti* (G. Lortet and C. Rosenberg, personal communication) and a *R. meliloti* mutant with a deletion which removes the *nodL* region exhibits slightly delayed nodulation

and produces NodRm-1 but not Ac-NodRm-1 (M. Y. Ardourel and C. Rosenberg, personal communication). These results indicate that Ac-NodRm-1 is also an active symbiotic signal and might be more active than NodRm-1.

*Rhizobium* sp. can elicit nodule organogenesis at a distance<sup>9,13-17</sup> and the involvement of an excreted bacterial 'nodule organogenesis inducing principle' (NOIP) which can diffuse through the plant cell wall has been hypothesized<sup>13</sup>. Our results confirm that nodule induction can be achieved by an excreted *R. meliloti* metabolite and indicate that NodRm signals are likely to be the NOIP which is produced by *Rhizobium in planta*. This hypothesis is supported by the fact that the only *R. meliloti* mutants unable to elicit nodule formation on alfalfa<sup>1,18-20</sup> (those altered in the *nodABC* or in the *nodH* genes) are simultaneously deficient in the production of NodRm-1 and Ac-NodRm-1 (refs 2, 4, 6 and 7). We thus propose that during the symbiotic process the production of NodRm signals by *R. meliloti* is involved not only in the induction of root-hair deformation and curling<sup>4</sup>, but also in the initiation of the first rounds of cortical cell division and in nodule organogenesis.

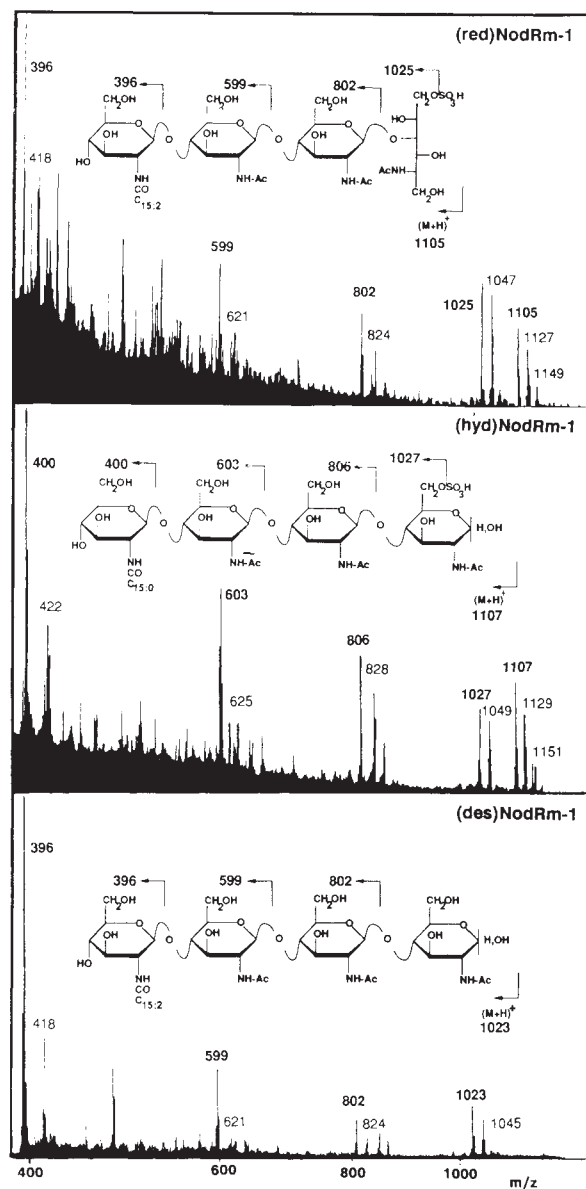
Oligosaccharides are known to elicit plant defence responses but there are only a few reports of their effect on plant morphogenesis<sup>21</sup>. For example, xyloglycan oligosaccharides have anti-auxin activity in pea<sup>22</sup> and pectin cell-wall fragments generated by treatment with endopolygalacturonase regulate tobacco thin-cell-layer explant morphogenesis<sup>23</sup>. But in these experiments oligosaccharides were not isolated directly from living plants but were derived from plant cell walls by chemical or enzymatic hydrolysis and thus the physiological significance of their effects is still hypothetical<sup>23</sup>. By contrast, NodRm-1 is specified by bacterial genes already shown to determine nodule formation and its morphogenic activity directly demonstrates that lipo-oligosaccharides can serve as signal molecules in plants and can induce a specific organogenesis. An important question is whether *Rhizobium* is mimicking an endogenous plant signalling mechanism, and hence whether lipo-oligosaccharide signals similar to NodRm-1 are synthesized in uninfected leguminous or non-leguminous plants.

In the system described here a chemically defined molecule produced by a procaryote induces a specific organogenesis. Nod factors have been shown to induce the transcription of host plant genes coding for proteins involved in early steps of symbiosis (refs 24 and 25; T. Bisseling, personal communication).



FIG. 4 Fast-atom bombardment mass spectrometry spectra of the chemically modified derivatives of NodRm-1. Mass numbers in bold characters refer to the protonated ions or to their fragment ion derivatives, whereas mass numbers in standard character refer to the sodium-containing ions. The positive FAB-MS of the NodRm-1 derivatives exhibited both pseudomolecular ions  $M+H^+$  and  $M+Na^+$ :  $m/z$  1,105 and 1,127 for (red)NodRm-1,  $m/z$  1,107 and 1,129 for (hyd)NodRm-1, and  $m/z$  1,023 and 1,045 for (des)NodRm-1. (hyd)NodRm-1 and (red)NodRm-1 showed fragment ions at 80 mass units lower than their  $M+H^+$  and  $M+Na^+$  pseudomolecular ions, corresponding to the elimination of  $SO_3$ . Additional cleavages of the interglycosidic bonds confirmed the sites of the chemical modifications. All these fragment ions contained the non-reducing end and the *N*-acylated part of the molecules. In particular, (red)NodRm-1 and (des)NodRm-1 possessed a common fragmentation pattern except at  $m/z$  1,025 and 1,023, respectively, thus showing that (red)NodRm-1 was only reduced at the reducing terminal glucosamine residue. By contrast, all the fragments were shifted up 4 mass units in the (hyd)NodRm-1 spectrum, corresponding to the hydrogenation of the fatty acyl chain. The high backgrounds observed with (red)NodRm-1 and (hyd)NodRm-1 spectra are likely to be due to acidic trace contaminants which were removed by ion-exchange chromatography from the nonacidic compound (des)NodRm-1. The structures of the modified NodRm-1 were also deduced from a comparison of their  $^1H$ -NMR spectra (data not shown) with those from NodRm-1 in ref. 7. (red)NodRm-1 was characterized by the disappearance of the signal assigned to the anomeric proton of the reducing sugar ( $\delta H=5.05$  p.p.m.). No downfield signals corresponding to the two double bonds of the acyl chain could be detected in (hyd)NodRm-1. Desulphation led to the disappearance of the three deshielded signals between 3.9 to 4.3 p.p.m. assigned to the H5, H6a and H6b of the sugar bearing the sulphate group (see ref. 7).

**METHODS.** NodRm-1 and Ac-NodRm-1 were prepared as already described from cultures of the overproducing *R. meliloti* EJ355 (pGMI149) strain by a combination of  $C_{18}$  reverse-phase HPLC, ion exchange and gel-permeation chromatography<sup>7</sup>. The mixture of NodRm-1 and Ac-NodRm-1 was treated with sodium methanolate to convert Ac-NodRm-1 into NodRm-1 as previously described<sup>7</sup>. At each step of the purification the presence and purity of NodRm-1 was tested using a biological assay (alfalfa root-hair deformation<sup>3</sup>) and physicochemical methods (FAB-MS and  $^1H$ -NMR spectrometry<sup>7</sup>). NodRm-1 was reduced by 1.0 M sodium borohydride in a 1:1 water:methanol mixture for 15 h at room temperature. The reduced product was purified on a reverse-phase  $C_{18}$  Sep-Pak cartridge<sup>7</sup>. Catalytic hydrogenation of NodRm-1 was carried out over Pd/C (Aldrich) in methanol for 1 h at 25 °C (ref. 7). To minimize possible contamination with unmodified NodRm-1, both reduction and hydrogenation were repeated twice. The desulphation of NodRm-1 was achieved by mild methanolysis in a 0.1 M HCl solution in dry methanol overnight at room temperature<sup>7,30</sup>. After neutralization, the desulphated derivative ((des)NodRm-1) was purified by HPLC on a DEAE column (4.6 × 150 mm; spherisorb 5 SAX; Biochrom) with a linear gradient from  $10^{-3}$  to  $5 \cdot 10^{-2}$  M ammonium acetate in ethanol and UV detection at 220 nm. Whereas the traces of NodRm-1 were retained on the column, (des)NodRm-1 was eluted and detected in the injection peak. Fast-atom bombardment mass spectrometry spectra were recorded on a VG-ZAB-BS instrument using an 8 KeV xenon atom beam. The matrix was a 1:1:0.01 mixture of *m*-nitrobenzylalcohol:glycerol:trifluoroacetic acid. The  $^1H$ -NMR spectra were obtained on a Bruker AC-200 spectrometer using  $CD_3OD$  as solvent.



The amenability of *Rhizobium* to the manipulation of Nod signals together with the availability of plant molecular markers for nodule formation should allow the study of how a signal molecule can be perceived, transduced and subsequently determine an important plant developmental switch. □

Received 4 April; accepted 9 May 1991.

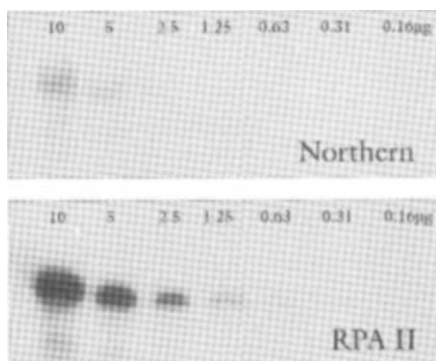
- Long, S. R. *Cell* **56**, 203-214 (1989).
- Fauchet, C. *et al. J. Bact.* **170**, 5489-5499 (1988).
- Fauchet, C., Camut, S., Dénarié, J. & Truchet, G. *Molec. Pl. Microbe Interact.* **2**, 291-300 (1989).
- Lerouge, P. *et al. Nature* **344**, 781-784 (1990).
- Schweidok, J. & Long, S. R. *Nature* **348**, 644-646 (1990).
- Roche, P. *et al. in Advances in Molecular Genetics of Plant-Microbe Interactions* Vol 1 (eds Hennecke, H. & Verma, D. P. S.) 119-126 (Kluwer Academic, Dordrecht, 1991).
- Roche, P., Lerouge, P., Ponthus, C. & Promé, J. C. *J. Biol. Chem.* (in the press).
- Dudley, M. E., Jacobs, T. W. & Long, S. R. *Planta* **171**, 289-301 (1987).
- Truchet, G. *et al. Molec. gen. Genet.* **219**, 65-68 (1989).
- Streeter, J. *C.R.C. crit. Rev. Pl. Sci.* **7**, 1-23 (1988).
- Spaark, H. *et al. in Advances in Molecular Genetics of Plant-Microbe Interactions* Vol 1 (eds Hennecke, H. & Verma, D. P. S.) 142-149 (Kluwer Academic, Dordrecht, 1991).
- Downie, J. A. *Molec. Microbiol.* **3**, 1649-1652 (1989).
- Truchet, G., Michel, M. & Dénarié, J. *Differentiation* **16**, 163-173 (1980).
- Finan, T. M. *et al. Cell* **40**, 869-877 (1985).
- Hirsch, A. M., Bhuvaneshwari, T. V., Torrey, J. G. & Bisseling, T. *Proc. natn. Acad. Sci. U.S.A.* **86**, 1244-1248 (1989).
- Hollingsworth, R., Squartini, A., Philip-Hollingsworth, S. & Dazzo, F. B. in *Signal Molecules in Plants and Plant-Microbe Interactions* (ed. Lugtenberg, B. J. J.) 387-393 (Springer, Berlin, 1989).
- Kapp, D., Niehaus, K., Quandt, J., Müller, P. & Pühler, A. *Pl. Cell* **2**, 139-151 (1990).
- Egelhoff, T. T. & Long, S. R. *J. Bact.* **164**, 591-599 (1985).
- Debellé, F. *et al. J. Bact.* **168**, 1075-1086 (1986).
- Horvath, B. *et al. Cell* **46**, 335-343 (1986).
- Albersheim, P. *et al. in Structure and Function of Plant Genomes* (eds Ciferri, O. & Dure, L. III) 293-312 (Plenum, New York, 1983).
- McDougall, G. J. & Fry, S. C. *Pl. Physiol.* **89**, 883-887 (1989).
- Eberhard, S. *et al. Pl. Cell* **1**, 747-755 (1989).
- Scheres, B. *et al. Cell* **60**, 281-294 (1990).
- Nap, J. P. & Bisseling, T. *Science* **250**, 948-954 (1990).
- Truchet, G., Camut, S., de Billy, F., Odorico, R. & Vasse, J. *Protoplasma* **149**, 82-88 (1989).
- Maillet, F., Debellé, F. & Dénarié, J. *Molec. Microbiol.* **4**, 1975-1984 (1990).
- Bovin, C., Camut, S., Malpica, C. A., Truchet, G. & Rosenberg, C. *Pl. Cell* **2**, 1157-1170 (1990).
- Sprent, P. *Applied Nonparametric Statistical Methods* (Chapman and Hall, London, 1989).
- Karlsson, K. A., Samuelson, B. E. & Steen, G. O. *J. Membrane Biol.* **5**, 169-184 (1971).

**ACKNOWLEDGEMENTS.** We thank F. Couderc and J. Roussel (CRBGC-CNRS, Toulouse) and the NMR Unit of the LCC-CNRS Toulouse for their help in physicochemical measurements, F. Maillet for help in the preparation of NodRm-1, A. Moisan (Station de Biométrie, INRA Toulouse) for the statistical analysis of nodulation assays and Fig. 3, and J. V. Cullimore and A. Stanford for reviewing the manuscript. This work was supported by the Conseil Régional Midi-Pyrénées.

# Choosing the right reagent

Stock up your shelves with the latest biochemicals and reagents to come off the production line, including new phage lambda cloning vectors, a genetically-engineered substrate for receptor-specific mammalian cell attachment, and a kit for detecting *Mycoplasma* contamination in cell lines.

AMBION announces the release of a new Ribonuclease Protection Assay (RPA II) Kit for **mRNA detection and quantitation** (Reader Service No. 100). The company claims that the RPA II Kit is at least ten times more sensitive than northern blotting and can detect many low abundance transcripts without poly(A) selection. RNase protection assays provide a sensitive and precise means of quantitating mRNA levels, mapping initiation and termination sites, and analysing mutation mismatches, says Ambion. The \$245 (US) RPA II Kit is a second-generation product that offers several improvements over the original RPA Kit. The net effect of



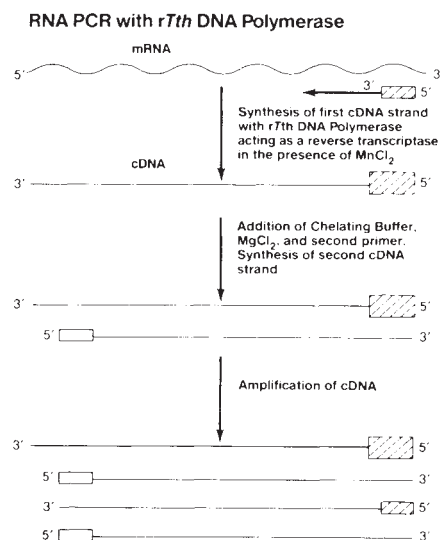
Amounts of total mouse liver RNA analysed by northern blotting and Ambion's RPA II kit. Both the blot and gel were exposed for 6 h.

the improvements has been a reduction in both the number of steps involved and in hands-on time. With the RPA II Kit, it is possible with moderately abundant messages to obtain results in a day. With high specific activity probes, as little as 100 femtograms of target RNA can be detected after a three-day exposure to X-ray film. The RPA II Kit includes a proprietary reagent that inactivates the RNases and precipitates the protected RNA in a single step. This eliminates the proteinase K digestion and phenol extraction that was required with the original kit, as well as the need to transfer the aqueous phase to a clean tube.

Novagen announces the availability of two new **phage lambda cloning vectors** (Reader Service No. 101). Both  $\lambda$ EXlox and  $\lambda$ SHlox vectors feature reliable automatic subcloning mediated by a site-specific recombinase, which allows conversion of phage to plasmid recombinants simply by plating on the appropriate host strain in the presence of ampicillin. Each vector has also been designed for specific applications. Inserts in  $\lambda$ EXlox can be expressed as part of a fusion

protein under control of the bacteriophage T7 promoter. Novagen says this vector is suitable for screening libraries with antibodies and for high-level protein production from plasmid subclones. In contrast,  $\lambda$ SHlox is suitable for all those applications that do not require protein expression, such as the synthesis of RNA probes and subtractive hybridization. The vectors are designed for efficient directional cloning of cDNA inserts into *Eco*R I and *Hind* III sites. Each is supplied as a \$225 (US) kit containing pretested vector arms, necessary host strains, a positive control and full documentation. Compatible cDNA and sequencing primers, directional linkers, and a methylation dNTP mix for cDNA synthesis are also available.

All the reagents that are necessary to **reverse transcribe RNA to cDNA** and subsequently amplify cDNA in a single reaction tube using the same enzyme are provided in the GeneAmp ThermoStable *rTth* Reverse Transcriptase RNA PCR Kit from Perkin-Elmer Cetus (Reader Service No. 102). The kit facilitates reverse transcription of RNA templates at elevated temperatures of up to 70°C and through regions that are G+C rich or regions that contain secondary structures. The method begins with the synthesis of first-strand cDNA with recombinant *Thermus thermophilus* (*rTth*) DNA polymerase acting as a reverse transcriptase in the presence of  $MnCl_2$ . Chelating buffer,  $MgCl_2$  and the second primer are then added. Synthesis of second strand cDNA then occurs,



Perkin-Elmer Cetus' kit uses one enzyme for reverse transcription and cDNA amplification.

which is followed by PCR amplification. The kit contains sufficient reagents to perform 100 reverse transcription reactions of 20  $\mu$ l each and 100 PCR amplifications of 100  $\mu$ l each.

According to Applied Biosystems, the **recovery of genomic DNA** from very small samples, such as 20  $\mu$ l of whole blood, tissue culture samples containing fewer than one-million cells and sub-milligram quantities of tissue, is now possible using the company's BaseBinder reagent (Reader Service No.



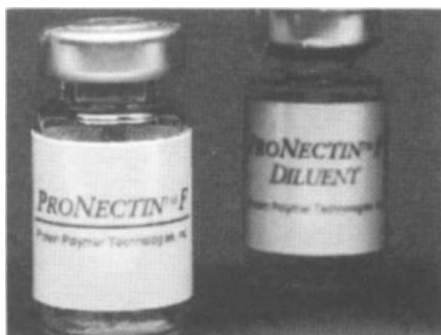
Genomic DNA recovery from small samples.

103). BaseBinder, which is composed of tiny silica spheres, is designed for use with AB's GENEPURE 341/340A system. The reagent is added after completion of the organic extraction and before DNA precipitation begins. After DNA non-covalently binds to BaseBinder, the reagent is trapped on a filter and the DNA eluted with water. As no additional cycle time is required, eight purifications can still be performed in 3.5 hours, says the company. Stated applications for BaseBinder include use in forensic science, biomedical research and other applications in which starting samples are small and PCR methods are used for the analysis of the genomic DNA recovered. Each vial contains sufficient BaseBinder for at least 50 purifications.

## Cell science selection

ProNectin F is the new highly stable **substrate for receptor-specific cell attachment** from Protein Polymer Technologies (Reader Service No. 104). This genetically engineered, high molecular weight protein polymer incorporates multiple copies of an extracellular matrix cell attachment ligand interspersed between repeated structural peptide segments. Due to both the design and method of production of ProNectin F, the problems commonly associated with the use of serum, animal-derived attachment





ProNectin F substrate for mammalian cell attachment.

factors and peptides for mammalian cell culture have been eliminated, says Protein Polymer Technologies. ProNectin F has been shown to exhibit a high degree of activity with a variety of mammalian cells and coat a variety of different substrates. Cells that have attached to ProNectin F include epithelial cells, fibroblast cells, endothelial cells, tumour cells, nerve cells, bone cells, muscle cells and parenchymal cells. Because of its engineered structure, the product is highly resistant to thermal and chemical denaturation, says the company. ProNectin F is sold in 1- and 5-mg kits for \$45 and \$180 (US), respectively, with the polymer powder and diluent in separate vials.

TransfectACE is a new reagent from Gibco BRL designed to provide a high efficiency method for **transfecting DNA** into BHK, HeLa or COS-7 cells for transient gene expression studies (Reader Service No. 105). The reagent is a 1:2.5 (w/w) liposome formulation of the cationic lipid dimethyldioctadecylammonium bromide and dioleoyl phosphatidylethanolamine in membrane-filtered water. TransfectACE works by interacting spontaneously with DNA to form a lipid-DNA complex, says Gibco BRL. Each 1ml of TransfectACE reagent is sufficient for approximately 25 to 30 DNA transfections on 60-mm tissue culture dishes, or 75 to 90 transfections on six-well plates when using BHK-21 cells. Gibco BRL notes that the volume of reagent required may vary with the cell line used.

TechGen International has introduced a range of **separation media** that is suitable for the one-step centrifugal isolation of lymphocytes, monocytes, polymorphonuclear granulocytes or platelets (Reader Service No. 106). The range includes J-Prep, which is designed for the preparation of pure lymphocytes for tissue typing; J.N. Prep 1.077, a special formulation that is suitable for the *in vitro* isolation of bone marrow cells; J.N. Prep 1.063 for the isolation of platelets from whole blood; J.P.M. Prep for the isolation of polymorphonuclear granulocytes; and J.N. Prep 1.068 for the isolation of monocytes from leucocyte-rich plasma or whole blood. J.N. Prep 1.077A, however, can be used in veterinary research to isolate mononuclear cells from animal species other than human.

The contamination of valuable cell lines by *Mycoplasma* can be the scourge of tissue culture laboratories. These organisms can grow up to  $10^8$  colony forming units per millilitre of cell culture supernatant, affecting cell growth, membrane characteristics and other cellular processes. **Cell-line contamination** by *Mycoplasma* can now be detected in three hours using the Gen-Probe Mycoplasma Detection Kit, according to Laboratory Impex, the company that distributes the kit (Reader Service No. 107). The kit, which uses a tritium-labelled DNA probe directed against the ribosomal RNA of *Mycoplasma* species, can be used to detect contamination in new cell lines, to screen regularly existing cell lines, and to monitor the progress of treatment.

### Gels and gel markers

Immobilized rProtein A from O.E.M. Concepts is designed for the **affinity purification of monoclonal antibodies** (Reader Service No. 108). The immobilized rProtein A is produced by coupling Ultrapure rProtein A to cross-linked six per cent agarose beads. The alkylamine coupling chemistry results in high binding capacities and very low levels of rProtein A leakage, says the company. Most human and mouse IgGs can be purified using immobilized rProtein A. The company claims that 1 ml of this ready-to-use preparation will bind approximately 10–20 mg of IgG, but notes that the binding capacity can vary for different subclasses and from antibody to antibody. Immobilized rProtein A is sold in quantities ranging from 5 ml to 1,000 ml; a 5-ml quantity costs \$120 (US).

BioMarker Low is a **double-stranded DNA molecular weight marker** from BioVentures that can be used to determine the molecular weight of DNA over the 50–1,000-base pair range (Reader Service No. 109). This dsDNA



electrophoresis marker provides fully resolved bands without ambiguity or comigration, says BioVentures. Bands are 50, 100, 200, 300, 400, 500, 700 and 1,000 bp, stain with equal intensity with ethidium bromide, and contain 50 ng per band. BioMarker Low is designed to permit the precise determination of product size and estimation of product yield. Plots of  $\log_{10}$  bp versus migration distance are linear, says BioVentures. BioMarker Low is available as a \$75 (US) 50-assay kit, which includes a 6× tracking dye and protocol. A trial-sized kit, which is sufficient for 20 assays, sells for \$40 (US).

### On the map

Lagan has launched two new LaganX.PLORER kits, each containing six

ADVERTISEMENTS

# DNA

**Around the World**

**\$4.50** a base  
(no set-up)

**Includes:**

- 500-1500 ugs
- 15-45 O.D.S.
- 24-72 hour delivery
- Amplification ready
- Gel picture
- Quality guaranteed products

**We are a full service and design DNA lab.**

**1-800-227-0627**  
**Fax 1-214-420-0442**

## BIO-SYNTHESIS

Reader Service No.18

### METABOLITES

analysed rapidly, enzymatically, using micro-samples

with the

#### ANALOX GM7 MICRO-STAT

A wide range of biological fluids including whole blood are acceptable for direct injection. Turbidity no problem. Sample size 2.5 - 25ul. Results in 20 seconds.

|             |          |               |
|-------------|----------|---------------|
| glucose     | lactate  | urea          |
| alcohol     | pyruvate | creatinine    |
| cholesterol | ammonia  | 3-OH butyrate |
| uric acid   |          | acetoacetate  |

**Analox Instruments Ltd**  
8 Goldhawk Industrial Estate  
Brackenbury Road  
Hammersmith, London W6 0BQ  
Tel: 081-749 7635  
Fax: 081-740 6608

Reader Service No.17

## Custom DNA

Purified and Shipped in 48 hours, Worldwide.

**100 ug, \$10.00 a base for the first 15 bases, \$7.50 for each additional base. Delivered.**

### Research Genetics

**1-800-533-4363**  
In the United Kingdom 0-800-89-1393  
In Canada 1-800-533-4363  
**FAX 205-536-9016**

Reader Service No.32

new and unique **DNA mapping probes** (1 µg each), which can be used in the mapping of human and higher primate genomic DNAs (*Reader Service No. 110*). The kits, each costing \$300 (US), can be used to map cloned genomic DNAs in YACs, phages and cosmids. Specifically, probes in each kit individually identify different sequences that are repeated 300 to 10,000 times throughout the human haploid genome. Consequently, the probes can be used in a procedure (included in the kits) that describes a method of identifying contigs that are useful in a more rapid mapping of genomic DNA.

ICI Pharmaceuticals has constructed a **yeast artificial chromosome (YAC) library** that could be a useful resource to medical



ICI's YAC library.

researchers (*Reader Service No. 111*). Use of the YACs in identifying and understanding the effects of disease-causing genes may lead to early diagnostic tests and treatments, ICI says. The YAC library has already proved to be a useful research tool. It was used for cloning the cystic fibrosis gene in 1989 and, more recently, YACS from the library played a key role in the isolation of one of the genes that may cause colon cancer in humans, says ICI. The YAC library consists of 35,000 individual clones, each containing an average of 35,000 base pairs of human DNA as a linear chromosome which can be propagated in a yeast cell.

The *Human Genes Oligonucleotides Handbook* from GENSET provides users with a centralized **oligo data bank** of over 1,500 of the company's oligos for research use (*Reader Service No. 112*). This useful reference tool is intended to offer researchers a comprehensive source of oligonucleotides for the study of human genes in the fields of genetic disease, oncology and polymorphism studies. For each molecular defect, classified according to its chromosomal alignment, the handbook provides an introductory map, a detailed note on the molecular points involved in the defect, and a panel of corresponding oligos with, for each oligo, the name, sequence, annealing temperature or calculated  $T_m$ , positions when aligned on the EMBL sequence data base or Genbank, literature references, and a map of each of the corresponding genes that gives an explicit view of the oligonucleotides. This handy guide costs FF100 (France).

### Antibody assortment

**Polyclonal antiserum to synaptophysin**, which recognises a single band with a molecular weight of 38 kD in immunoblot analysis of total rat brain homogenate, is now available from Biometra (*Reader Service No. 113*). In contrast to monoclonal antibodies, Biometra says the polyclonal antiserum reacts with all mammalian, fish and insect synaptophysin preparations that have been tested so far. These include synaptic vesicles of the central nervous system, chromaffin granules of the adrenal medulla, endocrine cells of both stomach and pancreas, and Type-C cells of thyroid and

secretory vesicles in the rat cell line PC12. In addition, the antiserum can be used for specific staining of neuronal, adrenal and neuroepithelial tumours. Antiserum to synaptophysin, which costs £170 (UK) for 300 µl, can be used with both immunoblot and immunohistochemistry procedures.

T Cell Sciences has introduced a **monoclonal antibody that recognises human granzyme A** in frozen tissue sections and fixed-cell preparations, and which can also be used for immunoprecipitation procedures (*Reader Service No. 114*). Granzyme A, which is found in the intracellular cytoplasmic granules of cytotoxic T lymphocytes, has a trypsin-like activity, is absent in resting T lymphocytes, but appears in T cells upon *in vitro* activation, says the company. Recent studies using *in situ* hybridization techniques have detected lymphocytes that express granzyme A RNA infiltrating the human donor heart after transplantation. These studies indicate that granzyme A can be identified in rejecting tissue biopsies before histological damage is apparent, and suggest that granzyme A may have a role in the cytotoxicity that occurs during cardiac transplant rejection.

### Off the shelf

Novex, a manufacturer of electrophoresis products for the separation of proteins and nucleic acids, has just released its 1991 Product Selection Guide (*Reader Service No. 115*). Over 100 **precast gels** are listed, vary-



Gel selection made simple — see Novex.

ing in composition, thickness and comb configuration. The catalogue features gels designed for specific applications, including PCR analysis, DNA retardation assays, and both proteinase and peptide analysis. The catalogue also includes a handy gel selection guide to help users match the appropriate gel to the right application.

The 1991 free **molecular biology product guide** from Sigma is now out (*Reader Service No. 116*). The guide contains an expanded listing of new chemicals and equipment arranged in a user-friendly format to speed up the ordering process. □

*These notes are compiled by Diane Gershon from information provided by the manufacturers. To obtain further details, use the reader service card bound inside the journal. Prices quoted are sometimes nominal, and apply only within the country indicated.*

#### ADVERTISEMENTS

#### Professional Custom Services

**DNA SYNTHESIS  
PROTEIN SEQUENCING  
PEPTIDE SYNTHESIS**

Rapid Service • Guaranteed Quality • Ample Quantity  
Competitive Prices • Free Quotations  
World-wide Delivery

**V.G. Services** 1200 Wonderland Rd. S. Bldg. #7, Unit #2  
London, Ontario Canada N6L 1A6  
CALL/FAX: (519) 652-3758 ALT. FAX: (519) 652-9622

Reader Service No.16

**Economical Tissue Culture Incubator**

- Safe for AIDS research • Reliable, for In-Vitro fertilization
- Versatile enough for thousands of lab uses

**Easy to use** — Just flush with gas mixture, seal and place at approp. temp. Perform multiple experiments simultaneously w/out cross contamination. Thousands in use worldwide.

For information contact: **Flow Laboratories, Ltd.** or **Billups-Rothenberg, Inc.** • P.O. Box 977  
Del Mar, CA 92014-0977 USA • (619) 755-3309

Reader Service No.37

#### Synthetic Oligonucleotides

Custom made probes and primers,  
synthesised, purified and shipped in 3 days

**£3.50 per base**

(All inclusive)

0.2 µM scale (approx. 500 µg)

Modified bases, biotin or digoxigenin labelling,  
data base analysis and full design facilities

Molecular Medicine Unit,  
King's College School of Medicine & Dentistry,  
Denmark Hill, London SE5 8RX

Tel. XX-44-(0)71 - 326 3126

FAX XX-44-(0)71 - 733 3877

Reader Service No.56